High Stem Cell Frequency in Acute Myeloid Leukemia at Diagnosis Predicts High Minimal Residual Disease and Poor Survival

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Abstract

Purpose: In CD34-positive acute myeloid leukemia (AML), the leukemia-initiating event originates from the $CD34^+CD38^-$ stem cell compartment. Survival of these cells after chemotherapy may lead to minimal residual disease (MRD) and subsequently to relapse. Therefore, the prognostic impact of stem cell frequency in CD34-positive AML was investigated.

Experimental Design: First, the leukemogenic potential of unpurified $CD34^+CD38^-$ cells, present among other cells, was investigated *in vivo* using nonobese diabetic/severe combined immunodeficient mice transplantation experiments. Second, we analyzed whether the $CD34^+CD38^-$ compartment at diagnosis correlates with MRD frequency after chemotherapy and clinical outcome in 92 AML patients.

Results: *In vivo* data showed that engraftment of AML blasts in nonobese diabetic/severe combined immunodeficient mice directly correlated with stem cell frequency of the graft. In patients, a high percentage of $CD34^+CD38^-$ stem cells at diagnosis significantly correlated with a high MRD frequency, especially after the third course of chemotherapy. Also, it directly correlated with poor survival. In contrast, total $CD34^+$ percentage showed no such correlations.

Conclusions: Both *in vivo* data, as well as the correlation studies, show that AML stem cell frequency at diagnosis offers a new prognostic factor. From our data, it is tempting to hypothesize that a large $CD34^+CD38^-$ population at diagnosis reflects a higher percentage of chemotherapy-resistant cells that will lead to the outgrowth of MRD, thereby affecting clinical outcome. Ultimately, future therapies should be directed toward malignant stem cells.

Acute myeloid leukemia (AML) is characterized by clonal growth and subsequent accumulation of myeloid blasts in the bone marrow. In most cases, patients can be treated effectively with chemotherapy schemes based on the combination of an anthracyclin and cytarabine. Chemotherapeutic treatment of AML results in a high percentage of complete remission (CR; ref. 1). However, despite these high CR rates, relapses occur frequently, resulting in an overall survival of only 30% to 40% at 4 years after diagnosis (1).

Relapse of AML is thought to originate from the outgrowth of persisting leukemic cells, called minimal residual disease (MRD; refs. 2–5). MRD can be detected in the majority of patients in (morphologic) CR using an immunophenotypical approach. To this end, leukemia-associated phenotypes are established at diagnosis, which can subsequently be used to identify MRD cells. The frequency of MRD cells persisting after chemotherapy has been found to have strong prognostic impact (2–5). It is thought that MRD cells resemble blasts at diagnosis (2) that survived chemotherapy and, therefore, likely originate from the stem cell compartment.

In CD34-positive AML, the $CD34^+$ leukemic stem cell is characterized by the absence of CD38 (6, 7), irrespective of French-American-British type (FAB), although there is debate on FAB M3 (8). It has been hypothesized that the $CD34^+CD38^-$ compartment contains the hematopoietic cells in which the leukemia-initiating event has occurred that results in AML (9). CD34 expression, including both primitive ($CD38^-$) and more differentiated ($CD38^+$) cells, has been proposed as a prognostic factor, although conflicting results have been described (reviewed in ref. 10).

These observations led us to hypothesize that a high frequency of $CD34^+CD38^-$ cells at diagnosis should result in a high MRD frequency after chemotherapy. Thereby, as MRD frequency itself has been shown to have strong prognostic impact, stem cell frequency at diagnosis should have direct prognostic impact.

To elucidate this, the frequency of $CD34^+CD38^-$ cells in AML patients at diagnosis was studied in 92 patients and correlated with MRD frequency after chemotherapy during CR.

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Materials and Methods

Patient samples at diagnosis. Bone marrow samples from patients with AML were obtained at the VU University Medical Center after informed consent at diagnosis and after chemotherapeutic treatment. In 16 cases at diagnosis, bone marrow samples were not available and peripheral blood was used. Diagnosis of patients was based on morphology using the FAB classification, immunophenotyping, and cytogenetics (11). Patient characteristics are shown in Table 1. Cells were analyzed fresh (n = 56) or after storage in liquid nitrogen (n = 36). In fresh samples, for immunophenotypical analysis, RBCs were lysed using Pharm lyse (BD Biosciences, San Jose, CA) and washed twice with PBS with 0.1% bovine serum albumin added. Frozen samples were prepared using a Ficoll gradient (1.077 g/mL; Amersham Biosciences, Freiburg, Germany) and a 10-minute RBC lysis on ice using 10 mL lysis buffer [155 mmol/L NH_4Cl, 10 mmol/L $KHCO_3$, 0.1 mmol/L Na_2EDTA (pH 7.4)]. Cells were then frozen in RPMI (Life Technologies, Paisley, United Kingdom) with 20% heat-inactivated fetal bovine serum (Greiner, Alphen a/d Rijn, the Netherlands) and 10% DMSO (Riedel-de Haen, Seelze, Germany) in isopropyl alcohol–filled containers and subsequently stored in liquid nitrogen. When needed for analysis, cells were thawed and suspended in RPMI with 40% fetal bovine serum at 37°C. Cells were washed and enabled to recover for 45 minutes in RPMI with 40% fetal bovine serum at 37°C. Cells were washed and suspended in PBS with 0.1% bovine serum albumin. The different treatment of samples, i.e., lysis alone for fresh samples or Ficoll isolation combined with lysis and freeze-thawing, likely affects only mature cells and not the blast compartment. Procedures were validated for both the use of fresh and frozen material. Therefore, six samples, two with high stem cell frequency, one median stem cell frequency, and three with low stem cell frequency, were analyzed fresh and after cryopreservation. No significant differences were found between fresh and frozen samples using Wilcoxon statistics (P = 0.3 for $CD38^-/CD34^+$). Also, both the median values for stem cell frequency and the range were quite similar in fresh (n = 56) and frozen (n = 36) samples, for $CD38^-/CD34^+$: 3.2% (0.1-82.5%) and 3.7% (0.1-95.1%), respectively. Therefore, results obtained by both procedures were combined.

Treatment characteristics. Patients were treated according to different treatment protocols defined by the Dutch-Belgian Hemato-Oncology Cooperative Group (HOVON). AML patients ≤60 years were treated according to the Dutch HOVON 29 (n = 27) and HOVON 42 (n = 38) protocols, AML patients ages >60 years were treated according to the Dutch HOVON 32 (n = 6) and HOVON 43 (n = 17) protocols (see www.hovon.nl for details), other patients (n = 4) were treated according to protocols modified from HOVON protocols. All protocols have basically an identical design, consisting of two remission induction cycles containing cytarabine followed by one consolidation cycle.

The HOVON 29 study was followed by the HOVON 42 study. The first cycle of both studies consisted of idarubicin and cytarabine. In the HOVON 42, patients were randomized to standard-dose or high-dose cytarabine. The second course consisted of amsacrin and cytarabine; again, for the latter, patients were randomized to different doses. Patients >60 years were treated according to HOVON 32 and 43 protocols. Induction cycles I and II of HOVON 32 consisted of cytarabine and granulocyte colony-stimulating factor with or without fludarabin. HOVON 43 started with an induction cycle containing cytarabine and low- or high-dose daunorubicin followed by a second-induction cycle with cytarabine. Patients under 60 years of age received either allogeneic (n = 15) or autologous (n = 7) stem cell transplantation or a course of intensive chemotherapy (n = 20) as postremission treatment.

Overall survival was calculated from date of diagnosis until date of death. Relapse-free survival was calculated from date of CR until date of death or relapse, with relapse defined as event. Finally, disease-free survival was calculated from date of CR until date of first event (i.e., death or relapse).

Table 1. Patient characteristics

No. patients	92
Male/female	45/47
Age at diagnosis, y, mean (range)	52 (16-77)
WBC count at diagnosis, 10^9/L, mean (range)	40 (1-349)
FAB classification, n (%)	
M_0	9 (10)
M_1	12 (13)
M_2	20 (22)
M_4	15 (16)
M_5	21 (23)
M_6	2 (2)
M_7	1 (1)
Refractory anemia with excess blasts	2 (2)
Refractory anemia with excess blasts in transformation	7 (8)
Not classified	3 (3)
Cytogenetic risk group, n (%)	
Favorable	13 (14)
Intermediate	59 (64)
Poor	12 (13)
No metaphases	8 (9)
Flt3ITD, n (%)	
Present	16 (17)
Absent	62 (67)
Not analyzed	14 (16)

Cytogenetics and Flt3 internal tandem duplications. Patients were classified in three risk groups for cytogenetic abnormalities according to Grimwade et al. (12). Favorable risk was defined by the presence of t(15;17) or t(8;21) or inv(16), and poor risk was defined by the presence of either five unrelated abnormalities, or monosomy 5 or 7, or abnormalities of the long arm of chromosome 5, or inv(3) (q21:q26). Patients who did not meet the criteria for poor or favorable risk were classified as intermediate risk.

FLT3 mutant allelic expression was examined by reverse transcription-PCR with R5 and R6 primers previously described by Nakao et al. (13). This primer set covers the transmembrane and juxtamembrane domains of the *FLT3* gene. PCR products were visualized by gel electrophoresis.

Transplantation of acute myeloid leukemia blasts in nonobese diabetic/severe combined immunodeficient mice. Engraftment of diagnosis AML blasts in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice was studied, in relation to the $CD34^+CD38^-$ and $CD34^+$ frequency of the transplant. Samples were cryopreserved, after Ficoll isolation and T-cell depletion using *S*-adenosyl-L-ethionine–treated sheep RBC. These procedures resulted in fractions with very high percentages of blasts. Samples were obtained from patients with M_0 (n = 2), M_1 (n = 2), M_2 (n = 5), M_4 (n = 5), and M_5 (n = 7). Before transplantation, CD34 and CD38 expression were determined using flow cytometry. After total body irradiation of the mice with 3.5 Gy γ-irradiation, 3×10^7 AML blasts, containing the total purified blast population, were injected in the lateral tail vein (14). The mice were killed after 30 days in accordance with the institutional animal research regulations. Chimerism was determined using flow cytometric detection of human CD45 expression combined with CD34, CD33, and CD38 expression. Criteria were defined for both chimerism and CD34 percentage. Chimerism should be >1% because the presence of AML blasts in the bone marrow of mice with chimerism <1% can be explained by simple sustenance of cells that homed to the bone marrow (15). Moreover, CD34 percentage had to be >1%, because in samples with a CD34 percentage

<1%, the $CD34^+$ cells are, in general, of normal origin (16). These criteria resulted in the exclusion of three samples because of lack of sufficient chimerism.

$CD34^+CD38^-$ frequency assessment using fluorescence-activated cell sorting analysis. Cells, prepared in PBS containing 0.1% bovine serum albumin, were incubated with monoclonal antibodies (mAb) for 15 minutes at room temperature, washed once in PBS containing 0.1% bovine serum albumin, and analyzed on the flow cytometer. mAb combinations are shown in Table 2. Combinations consisted of FITC-, peridinyl chlorophyllin–, or allophycocyanin-labeled mAbs. Anti-CD34 FITC, anti-CD34 peridinyl chlorophyllin, anti-CD45 peridinyl chlorophyllin, anti-CD45 allophycocyanin, anti-CD38 allophycocyanin, and Via-Probe (7-amino-actinomycin D) were all from BD Biosciences; anti-CD34 FITC was in part of the samples from Immunotech (Marseilles, France); and Annexin V FITC was from Nexins Research (Kattendijke, the Netherlands). Although different clones, both anti-CD34 FITC were IgG1 and class III. It is known that mAbs specific for class III CD34 epitopes detect similar numbers of $CD34^+$ cells in large arrays of specimens and, therefore, results could be combined (17). When frozen-thawed cells were used, Annexin V FITC was included in the majority of cases to gate out dead/apoptotic cells in the tube, which further contained anti-CD34 peridinyl chlorophyllin and anti-CD45 allophycocyanin. In a second tube containing Annexin V FITC, anti-CD34 peridinyl chlorophyllin, and anti-CD38 allophycocyanin, these apoptotic/dead cells were then gated out before stem cell frequency assessment. In the remaining part, a sensitive approach was applied using Syto16 (Molecular Probes, Eugene, OR) together with 7-amino-actinomycin D (18) in one tube, which enabled to identify apoptotic/dead cells based on low forward and sideward scatter properties. In a second tube containing anti-CD34 FITC, anti-CD38 allophycocyanin, and anti-CD45 peridinyl chlorophyllin, these apoptotic/dead cells were then gated out before stem cell frequency assessment. Data acquisition was done using a FACScalibur (BD Biosciences) equipped with an argon and red diode laser and analysis was done using Cellquest software (BD Biosciences).

Blasts were identified by CD45dim/SSC characteristics according to Lacombe et al. (19). The gating strategy used takes into account that the $CD34^+CD38^-$ population is a population with very low frequencies and is shown in Fig. 1. For the correlation analysis, the $CD34^+$ population was divided in two populations, $CD38^-$ and $CD38^+$, populations that were defined using PBS as a negative control. PBS could be used as a negative control because isotype controls offered the same results for the relevant antibodies (and clones) used in this study (2). The percentage of $CD34^+CD38^-$ cells was calculated in two different ways, first as part of the total blast population (i.e., CD45dim/SSClow) further called $CD38^-$/CD45dim, and second as part of the total $CD34^+$ population (i.e., CD45dim/SSClow/$CD34^+$) further called $CD38^-$/$CD34^+$. Similar to NOD/SCID experiments, only AML samples with a CD34% >1 were included in the analyses (16).

In a randomly selected part of the cases, anti-CD123 PE (BD Biosciences) was used in combination with anti-CD34 allophycocyanin, anti-CD38 FITC (BD Biosciences), and anti-CD45 peridinyl chlorophyllin to establish the leukemic nature of the stem cells (20). Stem cells were considered positive for CD123 if the total population showed a positive shift of fluorescence compared with PBS control.

Minimal residual disease detection. At diagnosis, leukemia-associated phenotypes were established according to the methods described by us before (2, 21). In follow-up samples, $\sim 1 \times 10^6$ bone marrow cells were incubated with mAb combinations that defined the leukemia-associated phenotype (2, 21). MRD was investigated in a situation of full bone marrow recovery after first and second course of chemotherapy and within 6 months after the third course of chemotherapy as previously described (2, 21). RBCs were lysed using fluorescence-activated cell sorting lysing solution (BD Biosciences); cells were washed twice in PBS with 0.1% bovine serum albumin, labeled with appropriate mAb combinations, washed again, and measured by flow cytometry. PBS was used as a negative control because isotype controls offered the same results in MRD analysis (2).

Statistical analyses. Statistical analysis of the data was done using the SPSS 9.0 software package (SPSS, Chicago, IL).

Cox regression analysis was done for initial screening of the correlations between both $CD34^+$ and $CD34^+CD38^-$ expression and patient prognosis and for determining the prognostic significance of WBC count and age. Kaplan-Meier analysis was used to study the prognostic impact of Flt3-ITD, cytogenetics, and CR rate and to study the effect of cutoff values for $CD38^-$/$CD34^+$ and $CD38^-$/CD45dim on both MRD frequency and clinical outcome in our patient group. The cutoff value for $CD38^-$/CD45dim or $CD38^-$/$CD34^+$ was chosen at a percentage that resulted in the largest difference in survival between the two groups defined by that cutoff. The log-rank test was used to validate equality of the survival distributions.

To obtain cutoff values, part of the samples was used as a training set ($n = 60$) and the most significant cutoff value was chosen. The thus obtained cutoff values were subsequently used as cutoff values for the whole set ($n = 92$) of samples.

Multivariate analysis, using Cox regression analysis, was used for the correlation of stem cell frequencies with survival parameters to test the independence of known prognostic risk factors. Missing value analysis was done for Flt3ITD and validated using Little's test for Missing Completely at Random. Using this approach, multivariate analysis was possible on the total group.

The Mann-Whitney U test for nonparametric samples was used to correlate $CD38^-$/$CD34^+$ and $CD38^-$/CD45dim with MRD frequency after different cycles of chemotherapy. Spearman correlation analysis was used for the correlation between the frequency of $CD34^+$ or $CD38^-$/$CD34^+$ and chimerism determined in NOD/SCID mice.

$P < 0.05$ was considered significant.

Results

Nature and biological properties of $CD34^+CD38^-$ cells. To establish the stem cell character of $CD34^+CD38^-$ cells, the

Table 2. mAb combinations

Sample	mAb	Label	Clone	mAb	Label	Clone	mAb	Label	Clone
Fresh	CD34	FITC	8G12 class III/581 class III	CD45	PerCP	2D1	CD38	APC	HB7
Frozen	Annexin V	FITC		CD34	PerCP	8G12	CD45	APC	2D1
	Annexin V	FITC		CD34	PerCP	8G12	CD38	APC	HB7
Frozen	Syto16			7-AAD					
	CD34	FITC	8G12 class III	CD45	PerCP	2D1	CD38	APC	HB7

NOTE: mAbs used were IgG1. 8G12 was from BD Biosciences and 581 was from Immunotech.
Abbreviations: APC, allophycocyanin; PerCP, peridinyl chlorophyllin; 7-AAD, 7-amino-actinomycin D.

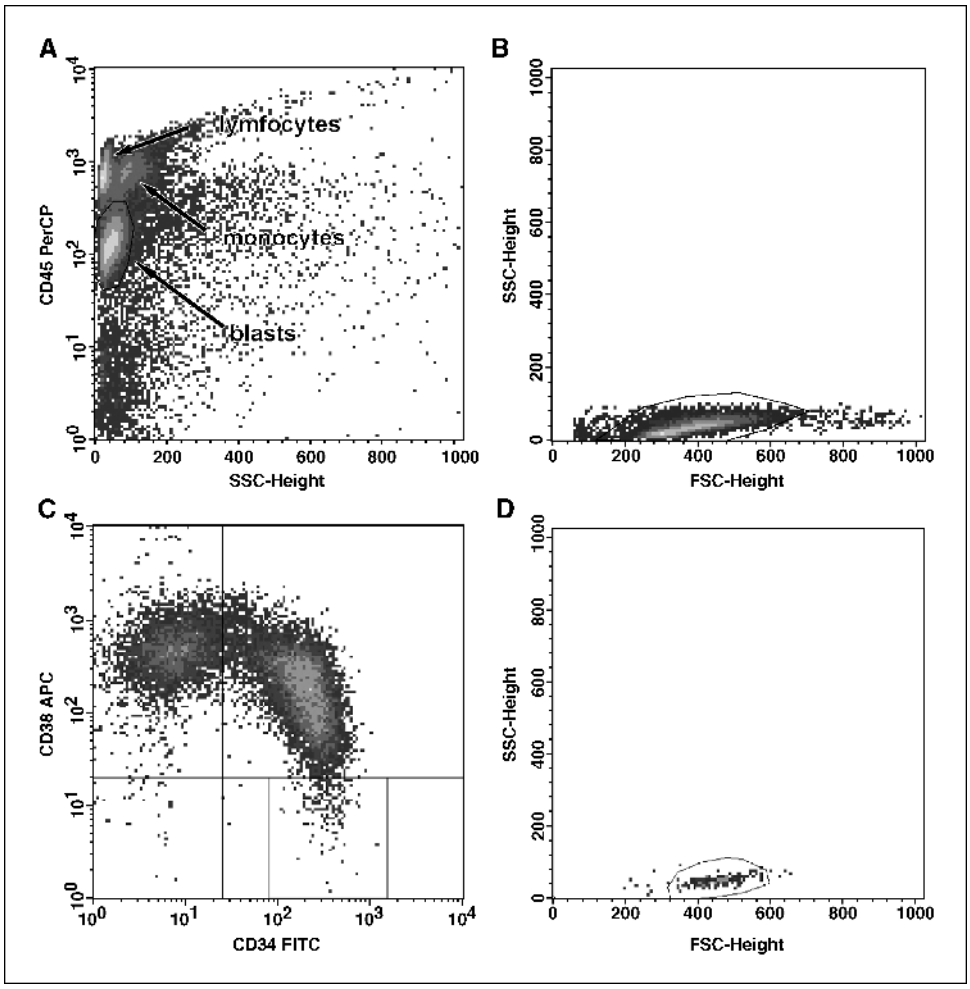

Fig. 1. Gating strategy in diagnosis AML to identify $CD34^+CD38^-$ cells. *A*, after labeling of diagnosis AML cells with the appropriate antibody combinations, the $CD34^+CD38^-$ cells were identified by a Boolean gating strategy. Gating on blasts characterized by CD45dim/low side scatter (*SSC*). *B*, gating of the blasts within the gate defined by forward scatter (*FSC*) and side scatter to identify a population that is roughly homogeneous for scatter properties. *C*, cells from the forward scatter/side scatter plot defined in (*B*) are shown in (*C*) in a plot defined by CD34 and CD38 expression. The $CD38^-$ population was defined using PBS as a negative control. *D*, the $CD34^+CD38^-$ population defined in (*C*) is back gated in a forward scatter/side scatter plot to identify a $CD34^+CD38^-$ population with homogeneous scatter properties. The frequency of the thus determined $CD34^+CD38^-$ population was used in this study.

thus defined $CD34^+CD38^-$ population was purified using fluorescence-activated cell sorting and examined in long-term culture assays (22). The $CD34^+CD38^-$ showed long-term clonogenic capacity in contrast to the $CD34^+CD38^+$ population (data not shown). The gating strategy that was used is shown in Fig. 1 and described in Materials and Methods.

To establish the nature (leukemic or normal) of the $CD34^+CD38^-$ stem cell compartment, CD123 expression (known to be present on leukemic stem cells but not on normal stem cells; ref. 20) was established in 36 consecutive cases. In 35 of 36 cases, all stem cells were positive, according to the criteria described under Materials and Methods, confirming the leukemic nature of the $CD34^+CD38^-$ cells. In the remaining CD123-negative sample, the leukemic nature of the $CD34^+CD38^-$ cells was confirmed using functional, P-glycoprotein activity (16), and phenotypical characteristics (i.e., the presence of a leukemia-associated phenotype on the $CD34^+CD38^-$ cells). P-glycoprotein activity of the $CD34^+CD38^-$ cells was 1.3, which is below the reference range of 1.7 to 3.7. The leukemia-associated phenotype of this patient (aberrant expression of CD56, a well-known aberrant phenotype; refs. 2–5) was also present on the $CD34^+CD38^-$ cells. To conclude, both functional and phenotypical characteristics confirmed the malignant nature of the $CD34^+CD38^-$ cells in this patient. Control bone marrow $CD34^+CD38^-$ cells ($n = 5$) were all CD123 negative.

We investigated engraftment of 21 AML samples. Stem cell frequencies were determined before transplantation, using flow cytometry. A fixed number of cells, representing the total blast population, were injected. Chimerism in NOD/SCID mice turned out to be strongly dependent on the stem cell frequency in the graft, with $P < 0.001$ for $CD38^-/CD34^+$ ($n = 21$, $R = 0.701$; Fig. 2A) and $P = 0.007$ for $CD38^-/CD45dim$ ($n = 21$, $R = 0.57$; data not shown), while engraftment was completely independent of total CD34 frequency (Fig. 2B). This indicates that the leukemia-initiating cell is present within the $CD34^+CD38^-$ compartment and, in this study, present among other cells, gives rise to leukemia in a dose-dependent way.

$CD34^+CD38^-$ cells and prognosis in acute myeloid leukemia. The putative prognostic value of the frequency of $CD34^+CD38^-$ cells at diagnosis was studied in 92 patients.

First, stem cell frequencies, defined either as $CD38^-/CD34^+$ or $CD38^-/CD45dim$ (for definitions, see Materials and Methods), were determined in a training set of samples of 60 newly diagnosed patients. A significant correlation was found between $CD38^-/CD34^+$ and all survival parameters (overall survival overall survival, relapse-free survival, and disease-free survival) and between $CD38^-/45dim$ and relapse-free survival and disease-free survival (Cox regression analysis). The most significant cutoff values, determined to identify two groups with the largest difference in survival, were 7.5% for $CD38^-/$

$CD34^+$ and 3.5% for $CD38^-$/CD45dim for all survival parameters (data not shown).

Next, the same correlations were examined for the complete group of 92 patients. $CD38^-/CD34^+$ correlated best with all survival parameters, overall survival (n = 92, P = 0.02), disease-free survival (n = 60, P = 0.06), and relapse-free survival (n = 60, P = 0.005) using Cox regression analysis. Figure 3A shows the results for $CD38^-/CD34^+$ and relapse-free survival for these 60 patients who fulfilled criteria for relapse-free survival in the total group of 92 patients. Using the cutoff values defined in the training set, results were best for $CD38^-$/CD45dim. Figure 3B shows results for $CD38^-$/CD45dim and relapse-free survival in a Kaplan-Meier plot, using the most significant cutoff value of 3.5% determined in the training set. Due to the particular shape of the Cox regression curves, like that shown in Fig. 3A, cutoff points are not based on an objective median value. However, it was nevertheless checked whether the most significant cutoff value was not the only significant P value. It is clear that only cutoff values to the right of the optimal cutoff value should be tested for this. Therefore, other cutoff values, to the right of 3.5% (for $CD38^-$/CD45dim), were tested for significance and this was found up to 30%. To conclude, high stem cell frequency was found to correlate with poor survival.

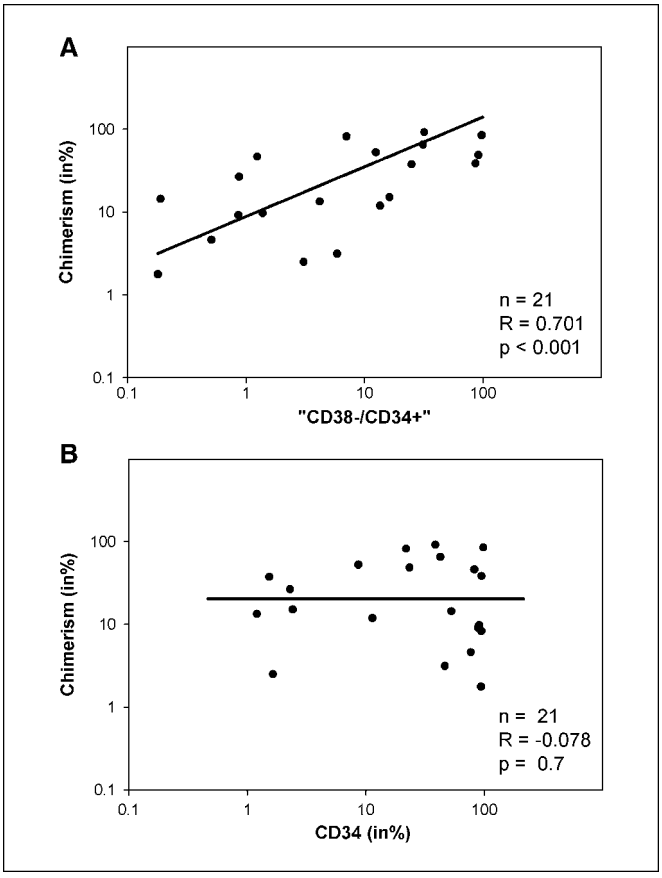

Fig. 2. Correlation between stem cell frequency of human AML blasts at diagnosis and chimerism in NOD/SCID mice. Equal numbers of AML blasts from 21 diagnosis samples were injected into NOD/SCID mice as described in Materials and Methods. *A*, engraftment (depicted as the percentage of chimerism, which is a measure for the engraftment of human AML cells in a NOD/SCID mouse) is dependent on the stem cell frequency ($CD38^-/CD34^+$) in the AML bone marrow sample at diagnosis. *B*, engraftment is not related to the absolute number of injected $CD34^+$ cells.

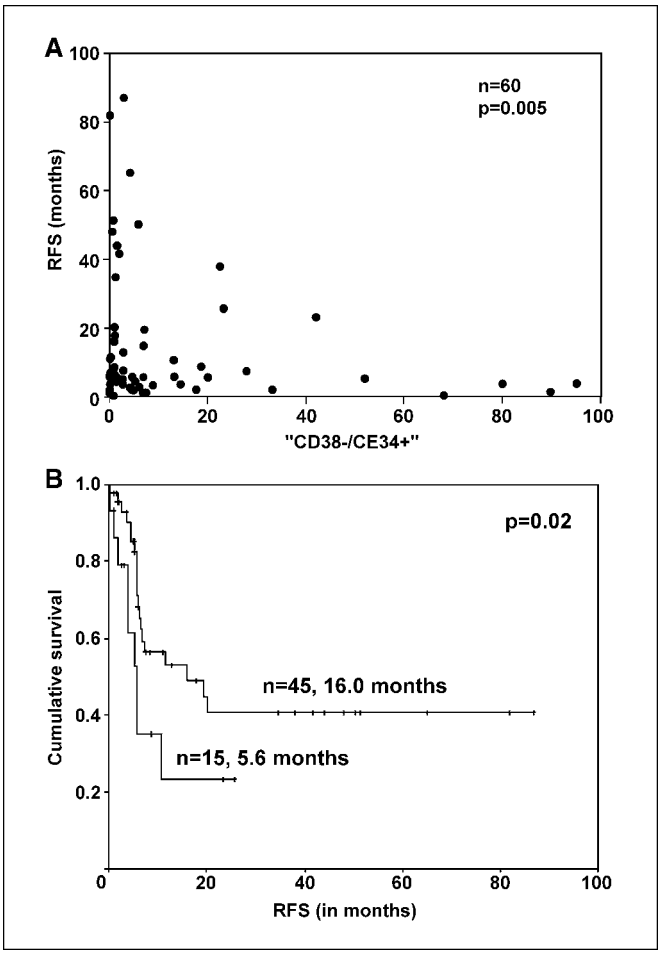

Fig. 3. Correlation between stem cell frequency and relapse-free survival (*RFS*). *A*, relation between the stem cell frequency $CD38^-/CD34^+$ and relapse-free survival (in months) for all 60 patients of the total group of 92 patients, who fulfilled criteria for relapse-free survival. Cox regression analysis showed a significant correlation (P = 0.005). *B*, a Kaplan-Meier plot of the same patients but now $CD38^-$/CD45dim with relapse-free survival. The cutoff used is 3.5%. The patients with a high stem cell frequency at diagnosis (>3.5%) had a median relapse-free survival of 5.6 months (n = 15). This is in strong contrast to the patients with a low stem cell frequency who had a median relapse-free survival of 16.0 months (n = 45). This difference was significant using log-rank statistics (P = 0.02).

As there are conflicting data in literature about the impact of CD34 expression on prognosis, total CD34 expression was also analyzed in this study with regard to survival parameters using Cox regression analysis. No significant correlations were found between CD34 expression, both with overall survival (n = 92, P = 0.26), disease-free survival (n = 60, P = 0.45), and relapse-free survival (n = 60, P = 0.13).

Finally, CR rate was analyzed in the groups defined by low and high stem cell frequency. Only a trend toward high stem cell frequency in the non-CR group was apparent (for $CD38^-/CD34^+$, P = 0.20, n = 84). CR rate showed no correlation with total CD34 expression (P = 0.14, n = 84).

Risk factors, such as WBC count at diagnosis, age, CR rate, and Flt3-ITD status were found to correlate with overall survival, showing that our patient cohort is representative for AML patients (not shown). Cytogenetics showed no prognostic significance in our study, probably due to small sample size in the poor and favorable group (Table 1).

In a multivariate analysis, results for relapse-free survival with $CD38^-/CD34^+$ ($P = 0.004$) were independent of WBC, age, and Flt3ITD ($P > 0.3$ for all variables). Results for disease-free survival with $CD38^-/CD34^+$ ($P = 0.05$) were also independent of WBC, age, and Flt3ITD ($P > 0.2$ for all variables).

Because CD123 expression is high on $CD34^+CD38^-$ cells in 35 of 36 cases, correlations with survival were almost similar (not shown).

To conclude, a high stem cell frequency at diagnosis correlates with poor survival, independent of known prognostic risk factors.

Correlation of $CD34^+CD38^-$ frequency with minimal residual disease frequency. Minimal residual disease (MRD) frequency, as detected after different cycles of chemotherapy, is a strong independent prognostic risk factor for survival, in particular after the third cycle of chemotherapy (2). In the present study, this could be confirmed using the same cutoff values as reported in the study we did before (data not shown; ref. 2).

Therefore, the prognostic impact on survival of stem cell frequency at diagnosis might be mediated by its direct correlation with MRD frequency after chemotherapy. This analysis was done using the same cutoff values defined in our correlation with survival parameters (3.5% for $CD38^-/CD34^+$ and 7.5% for $CD38^-$/CD45dim). Indeed, stem cell frequency at diagnosis was found to correlate with MRD frequency after the third cycle of chemotherapy in CR, with borderline significance for $CD38^-/CD34^+$ ($P = 0.05$; Fig. 4A) and significance for $CD38^-$/CD45dim ($P = 0.03$; Fig. 4B). No correlations were found between stem cell frequency at diagnosis and MRD frequency after the first course of chemotherapy. After the second cycle, a trend toward a higher MRD frequency in the group defined by a high stem cell frequency at diagnosis was observed. No correlations were found between total CD34 expression and MRD frequency ($P > 0.3$ for all three cycles).

Discussion

Relapse of AML is thought to originate from the outgrowth of persisting leukemic cells, called MRD (2–5). In the present study, we show that the $CD34^+CD38^-$ compartment in CD34-positive AML is of prognostic significance, with regard to overall survival, disease-free survival, and relapse-free survival. A high stem cell frequency at diagnosis also significantly correlated with a high MRD frequency, thereby providing the direct link with survival. No major correlations with CR were found. These results were independent of known risk factors. To the best of our knowledge, no studies have been done that address the prognostic impact of the frequency of the stem cell population in AML, although there have been studies on the prognostic impact of the level of CD38 expression, without taking into consideration the expression of CD34 (23).

Previously, using purified subpopulations, it has been established that SCID leukemia-initiating cells in CD34-positive AML are contained within the primitive $CD34^+CD38^-$ fraction (6). Although the issue was not specifically addressed, Monaco et al. (24) found, when using purified $CD34^+$ blasts, the highest engraftment in NOD/SCID mice transplanted with samples from two patients with the highest stem cell frequency. In accordance with this, we found that the stem cell frequency in unpurified AML samples strongly correlated with engraftment in the NOD/SCID mouse experiments.

There is evidence that leukemic stem cells, compared with more mature blasts, are difficult to eradicate in patients using conventional chemotherapy, due to their chemotherapy-resistant character (25). This is probably due to the fact that most of these cells are quiescent (in G_0 phase), which has been shown for normal $CD34^+CD38^-$ cells, when compared with normal $CD34^+CD38^+$ cells (26), and for AML (27). It thus seemed obvious to hypothesize that, with a relatively large number of intrinsically resistant $CD34^+CD38^-$ cells at diagnosis, the stem cell frequency in remission should be relatively high. Due to the very low frequency of $CD34^+CD38^-$ cells in remission bone marrow, together with their similarities with normal $CD34^+CD38^-$ stem cells, the leukemic stem cells are difficult to study in follow-up samples. However, the progeny of these stem cells, called MRD, often have different degrees of differentiation and with immunophenotypical make-up quite comparable with blasts at diagnosis. MRD can be studied more easily, using leukemia-associated phenotype technology: Immunophenotypical aberrances (leukemia-associated phenotype) detected at diagnosis are used to establish MRD frequency, which, in turn, has been shown to have strong prognostic impact (2–5, 28). It turned out that there was no

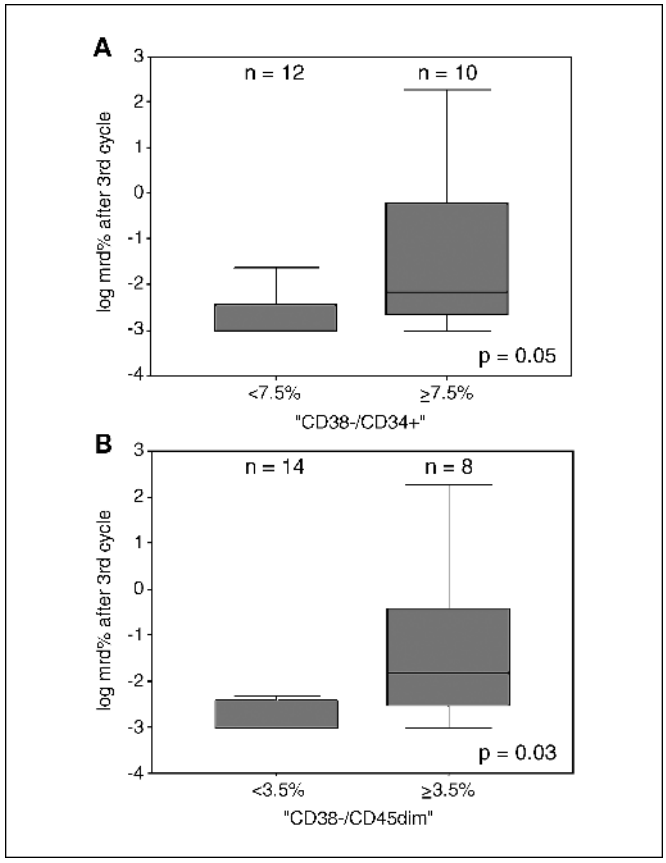

Fig. 4. Correlations between stem cell frequency at diagnosis and MRD frequency after third cycle of chemotherapy. *A*, patients were grouped according to the $CD38^-/CD34^+$ stem cell frequency of <7.5% and ≥7.5%, for which corresponding log-transformed MRD frequency after third cycle of chemotherapy is depicted ($n = 22$, $P = 0.05$). *B*, patients were grouped according to the $CD38^-$/CD45dim stem cell frequency of <3.5% and ≥3.5%, for which corresponding log-transformed MRD frequency after third cycle of chemotherapy is depicted as well ($n = 22$, $P = 0.03$). The box-and-whisker plots show in the box the median and the 25th and 75th percentile, while the whiskers show the 2.5th and 97.5th percentile.

correlation between stem cell frequency at diagnosis with MRD frequency after the first course of therapy. This is probably due to the least predictive value of MRD frequency for survival after first cycle. However, correlations became evident after subsequent courses: A higher stem cell frequency at diagnosis significantly correlated with a higher MRD frequency. The observation that MRD frequency after the second and third course of chemotherapy correlates most strongly with clinical outcome (2) gives further support for a direct causal relationship between stem cell frequency at diagnosis and MRD frequency.

Our observations are thus compatible with a model in which a higher stem cell frequency at diagnosis has no major impact on the CR rate and MRD frequency after one course of chemotherapy. After all, major leukemic cell reduction, and not reduction of the low-frequency stem cell fraction, likely determines CR rate. With additional courses of chemotherapy, the most resistant fractions of the $CD34^+CD38^-$ compartment will be selected. These resistant $CD34^+CD38^-$ cells will grow out, resulting in the spectrum of populations, referred to in all studies as MRD. The frequencies of these MRD cells reflect the resistant $CD34^+CD38^-$ frequencies. Depending on these frequencies, relapse will occur. The hypothesized direct relationship of $CD34^+CD38^-$ frequency at diagnosis with *in vivo* outgrowth in patients has been confirmed in NOD/SCID mice experiments, in which $CD34^+CD38^-$ frequency correlated with leukemogenic potential.

In AML other prognostic factors have been proposed, such as apoptosis and multidrug resistance (29–34). Multidrug resistance at diagnosis as well as apoptosis-related protein profile at diagnosis have been proven to be strong independent prognostic risk factors. If P-glycoprotein activity and Bcl-2 expression are both high in patients with high stem cell frequency, this could provide a molecular basis for our findings, which needs to be elucidated in the future. When defining new prognostic factors for future use in diagnostics, simple assays that can be done in routine settings are required. Assessment of the presence and size of a $CD34^+CD38^-$ population at diagnosis is easy to incorporate, if not already, in current diagnostic immunophenotypical protocols.

In many studies, the total $CD34^+$ fraction is still considered to be the progenitor/stem cell fraction of interest and although the number of $CD34^+$ cells has indeed been shown to be a prognostic risk factor in some studies (35, 36); others could not confirm that finding (37–39). In our patient cohort, a complete lack of correlation was found between $CD34^+$ frequency at diagnosis on the one hand and CR rate, MRD frequency, and clinical outcome on the other. Similarly, in the NOD/SCID mouse studies, the total number of $CD34^+$ cells had no predictive value for leukemogenic outgrowth.

A particular FAB class of leukemia in which the stem cell frequency has been addressed is FAB M_0: These have a higher stem cell frequency and poor prognosis compared with the other FAB types (40, 41). However, we were not able to confirm these data in our study, probably due to only nine patients with AML M_0.

In the future, it would be of great interest to study the frequency of $CD34^+CD38^-$ cells during follow-up. MRD detection is thus far mainly based on the presence of blasts with primitive markers (CD117 and/or CD133 and/or CD34) combined with aberrant marker expression. In CD34-positive AML, however, such blasts represent, in most cases, a more mature population compared with the $CD34^+CD38^-$ stem cells. In at least part of the patients, the aberrances are also found on $CD34^+CD38^-$ cells at diagnosis, but not on normal bone marrow stem cells.[3] In principle, this would allow to correlate stem cell frequency at diagnosis with stem cell frequency in CR after chemotherapy. Next, it would be of value to study if the prognostic impact of the MRD stem cell frequency might improve the already strong impact of total MRD frequency on outcome. Apart from leukemia-associated phenotype expression, the leukemia stem cell–selective expression of CD123 (20) and of the recently described C-type lectin–like molecule 1 (42) should be very helpful in this respect.

In CD34− AML, the compartmentalization of stem cells is still unknown. As ~20% of AML samples in our clinic are completely CD34 negative, more effort is needed to identify stem cells in CD34-negative AML.

Eradication of malignant stem cells can probably be reached in part of the patients, using conventional chemotherapy, e.g., via the application of repetitive cycles of consolidation therapy (43). However, given the prognostic significance of $CD34^+CD38^-$ cells in AML and the apparent difficulties in eradicating malignant stem cells from the patient, it will be necessary to develop therapies that specifically target these malignant stem cells after reaching CR. The possibility to detect and characterize stem cells will help to reach this goal. There is emerging evidence that such therapies are needed to offer curative possibilities for patients with AML.

[3] A. van Rhenen, A. Kelder, N. Feller, A.B. Bakker, B. Moshaver, A.H. Westra, G.J. Ossenkoppele, and G. Schuurhuis, unpublished data.

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