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Splenic pooling and loss of VCAM-1 causes an engraftment defect in patients with myelofibrosis after allogeneic hematopoietic stem cell transplantation

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ABSTRACT

Myelofibrosis is a myeloproliferative neoplasm that results in cytopenia, bone marrow fibrosis and extramedullary hematopoiesis. Allogeneic hematopoietic stem cell transplantation is the only curative treatment but is associated with a risk of delayed engraftment and graft failure. In this study, patients with myelofibrosis (n=31) and acute myeloid leukemia (n=31) were analyzed for time to engraftment, graft failure and engraftment-related factors. Early and late neutrophil engraftment and late thrombocyte engraftment were significantly delayed in patients with myelofibrosis as compared to acute myeloid leukemia, and graft failure only occurred in myelofibrosis (6%). Only spleen size had a significant influence on engraftment efficiency in myelofibrosis patients. To analyze the cause for the engraftment defect, clearance of hematopoietic stem cells from peripheral blood was measured and immunohistological staining of bone marrow sections was performed. Numbers of circulating $CD34^+$ were significantly reduced at early time points in myelofibrosis patients, whereas $CD34^+CD38^-$ and colony-forming cells showed no significant difference in clearance. Staining of bone marrow sections for homing proteins revealed a loss of VCAM-1 in myelofibrosis with a corresponding significant increase in the level of soluble VCAM-1 within the peripheral blood. In conclusion, our data suggest that reduced engraftment and graft failure in myelofibrosis patients is caused by an early pooling of $CD34^+$ hematopoietic stem cells in the spleen and a bone marrow homing defect caused by the loss of VCAM-1. Improved engraftment in myelofibrosis might be achieved by approaches that reduce spleen size and cleavage of VCAM-1 in these patients prior to hematopoietic stem cell transplantation.

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Introduction

Myelofibrosis (MF) is a rare chronic myeloproliferative neoplasm with an incidence of 0.22 to 0.99 per 100,000.[1] MF appears *de novo* or as a progression of polycythemia vera (PV) or essential thrombocythemia (ET). MF is characterized by bone marrow (BM) fibrosis, extramedullary hematopoiesis with splenomegaly, and severe constitutional symptoms.[2] As the prognosis of MF is heterogeneous, the Dynamic International Prognostic Scoring System (DIPSS) is widely used to stratify newly diagnosed MF patients prior to evaluation of therapy.[3]

Treatment options are conventional drugs including anti-proliferative medication, immunomodulatory drugs (iMiDs), Janus kinase (JAK) inhibitors and hematopoietic

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growth factors. However, allogeneic hematopoietic stem cell transplantation (HSCT) is the only curative treatment that is recommended to transplant-eligible intermediate 2 and high-risk patients.[4]

A number of studies have reported on the successful use of HSCT following reduced-intensity conditioning (RIC) that induces lower treatment-related mortality than myeloablative conditioning (MAC).[5,6] However, graft failure (GF) of up to 10% in MF patients after RIC HSCT is a critical contributor to morbidity and mortality.[7] Factors favorably affecting engraftment were shown to be splenectomy before transplantation, human leukocyte antigen (HLA) matched sibling donor, peripheral stem cell use and absence of pre-transplant thrombocytopenia.[8,9] The reconstitution of the BM after transplantation depends on the successful homing of transplanted hematopoietic stem

Table 1. Patients' characteristics at the time of transplant.

Myelofibrosis (MF) patients (n=31)		Acute myeloid leukemia patients (n=31)	
Median age (range), years	54 (36-67)	Median age (range), years	56 (37-68)
Female, n (%)	13 (42)	Female, n (%)	16 (52)
Disease, n (%)		Time of allo HSCT, n (%)	
Primary MF	17 (55)	Upfront after induction/consolidation therapy	15 (48)
Secondary MF	14 (45)	Relapse	16 (52)
JAK2V617F mutation status*			
Positive	5 (16)		
Negative	5 (16)		
Pre-treatment**			
Hydroxyurea only	13 (42)		
Hydroxyurea + additional therapy	7 (23)		
Other pre-treatment	3 (10)		
No cytotoxic treatment	8 (25)		
Response at time of transplant			
Progressive disease	16 (52)		
Stable disease	6 (19)		
Partial remission	1 (3)		
DIPPS, n (%)			
Low	3 (10)		
Intermediate-1	3 (10)		
Intermediate-2	22 (71)		
High	2 (6)		
Unknown	1 (3)		
Spleen size, n (%), median 21 cm (measured by ultrasonography)			
Greater than median	13 (42)		
Smaller than median	13 (42)		
Splenectomy, n (%)	5 (16)		
Bone marrow fibrosis according to Bauermeister scoring, n (%)			
Grade 1	3 (10)		
Grade 2	4 (13)		
Grade 3	6 (16)		
Grade 4	18 (58)		
Median number of transplanted $CD34^+$cells/kg/BWx10^6, n (range)	5.81 (3.53-8.99)	Median number of transplanted $CD34^+$cells/kg/BWx10^6, n (range)	5.98 (2.71-8.57)
Conditioning, n (%)		Conditioning, n (%)	
Myeloablative	4 (13)	Myeloablative	1 (3)
Reduced intensity	27 (87)	Reduced intensity	30 (97)
Antithymoglobulin	29 (94)	Antithymoglobulin	27 (87)
Donor, n (%) (on the basis of high HLA resolution testing: HLA-A, -B, -C, -DRB1 and-DQB1)		Donor, n (%)	
Related	8 (26)	Related	4 (13)
Unrelated matched	16 (52)	Unrelated matched	18 (58)
Unrelated mismatched	7 (22)	Unrelated mismatched	9 (29)
Major ABO mismatch, n (%)	11 (35)	Major ABO mismatch, n (%)	8 (26)
GvHD prophylaxis, n (%)		GvHD prophylaxis, n (%)	
CSA + MTX	18 (58)	CSA + MTX	20 (65)
CSA + MMF	10 (32)	CSA + MMF	10 (32)
CSA alone	3 (10)	Everolimus	1 (3)

*HSCT: hematopoietic stem cell transplantation; DIPPS: Dynamic International Prognostic Scoring System; BW: body weight; GvHD: graft-versus-host disease; CSA: cyclosporin; MTX: methotrexate. *JAK2V617F mutation was routinely tested after 2006. ** No pre-treatment with JAK2 inhibitor (JAK2 inhibitor was not available at this time).*

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cells (HSC).[10] After leaving the peripheral blood (PB), the HSC lodge into the stem cell niche that was shown to regulate the HSC pool.[11] Several cellular components were identified to regulate the hematopoietic homeostasis, among them endothelial cells, mesenchymal stroma cells and osteoblasts.[12,13] The latter were shown to express stromal cell-derived factor (SDF)-1 (CXCL12) and osteopontin (OPN) within the BM that control homing, quiescence and proliferation of HSC after transplantation.[12,14] In MF, the BM microenvironment is modified by fibrosis, osteosclerosis and neo-angiogenesis. BM fibrosis is the result of the abnormal deposition of collagen produced by fibroblasts that are stimulated by pro-inflammatory cytokines and growth factors.[15,16] Therefore, it can be speculated that the disarrangement of the BM niche in MF is one aspect of GF.[17]

In this study, we investigated neutrophil and platelet engraftment in patients with MF and acute myeloid leukemia (AML) following RIC-HSCT. In addition, factors that affect engraftment were evaluated. By measuring the number of circulating HSC at defined time points after transplantation we assessed the homing efficiency in patients with MF and AML. Finally, we analyzed BM extracellular components including chemokines and their receptors expressed on HSC within fibrotic and non-fibrotic BM.

Methods

Patients', disease and transplantation characteristics

A total of 31 patients diagnosed with MF and 31 age- and gender-matched patients with AML who underwent allogeneic HSCT in our department between 2000 and 2011 were retrospectively analyzed. Patients' and disease characteristics, as well as donor and transplant procedures are summarized in Table 1. This study was approved by the local ethics committee (n. 02/220). For further information see the *Online Supplementary Appendix.*

Clearance of HSC and colony-forming cells from the PB after HSCT

Clearance of $CD34^+$, $CD34^+CD38^-$ cells and colony-forming cells (CFC) from PB was measured at defined time points after infusion by flow cytometry and CFC assay from 5 MF and 5 AML patients. PB samples (2.5 mL) were taken prior to and 10, 20, 40, 80, 160 minutes (min), 6 and 22 hours (h), 3 days (d), and in some cases 5 d after transplantation.

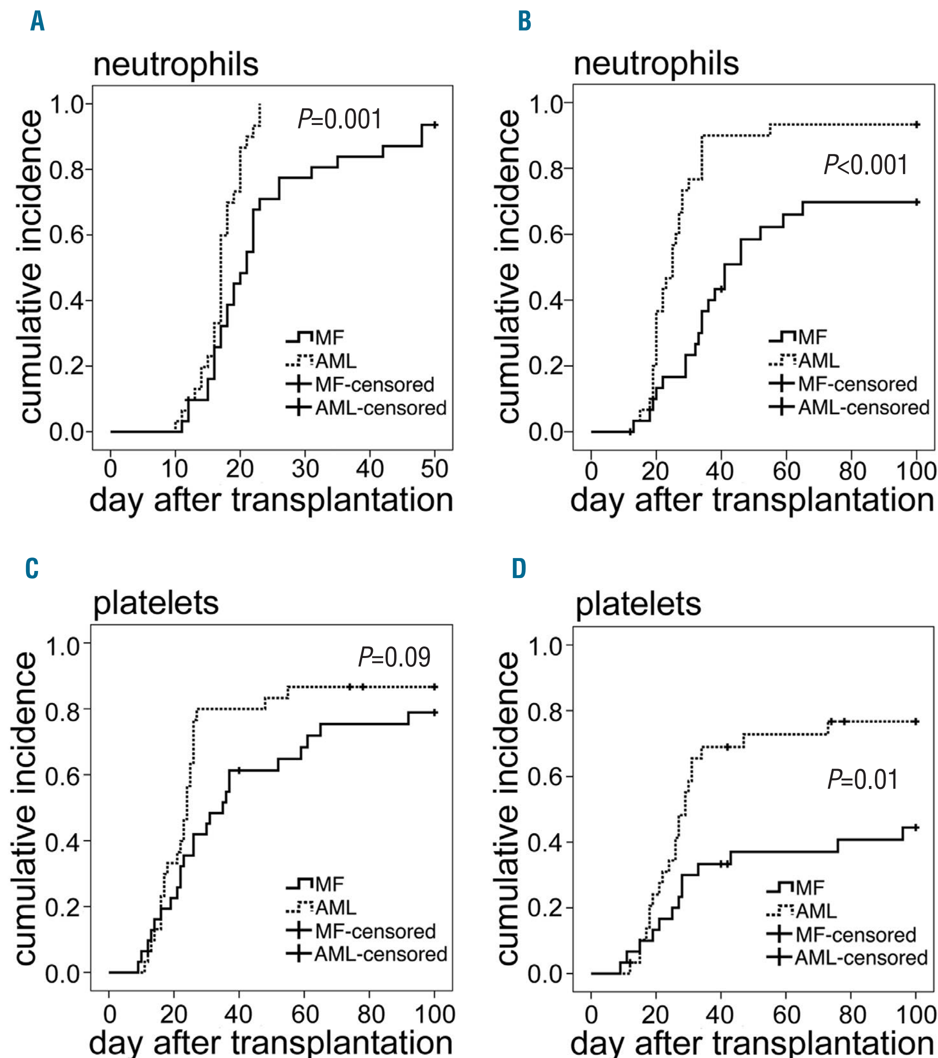

Figure 1. Myelofibrosis (MF) patients show significant delayed early and late engraftment as compared to acute myeloid leukemia (AML) patients. Cumulative incidence of early (A) and late (B) neutrophil and early (C) and late (D) platelet engraftment in MF and AML patients.

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Table 2. Characteristics of the myelofibrosis patients with primary or secondary graft failure.

Primary graft failure	Conditioning	Type of donor	ABO blood group	Spleen size (cm)
Patient # 1	RIC	MMUR	Minor mismatch	11
Patient # 2	RIC	MUR	Ident	> 20
Secondary graft failure				
Patient # 1	MAC	MUR	Major mismatch	26
Patient # 2	RIC	MUR	Major mismatch	40
Patient # 3	RIC	MRD	Ident	28
Patient # 4	RIC	MUR	Major mismatch	20
Patient # 5	RIC	MRD	Major mismatch	- (splenectomy)

MAC: myeloablative conditioning; RIC: reduced-intensity conditioning; MRD: matched related donor; MUR: matched unrelated donor; MMUR: mismatched unrelated donor. ; Ident: identical.

Flow cytometric analysis of circulating HSC

To analyze the $CD34^+$ and $CD34^+CD38^-$ cell count, PB was lysed in NH4CL lysis buffer and cells were then stained for 30 min at 4°C with combinations of anti-CD45-FITC, anti-CD34-APC and anti-CD38-PE monoclonal antibodies. Analysis was made using a Becton Dickinson CALIBUR flow cytometer (BD, East Rutherford, NJ, USA). Detailed information is provided in the *Online Supplementary Appendix.*

CFC assay

To measure the colony forming ability of transplanted cells, 1 mL of PB was processed as described above. Burst-forming and colony-forming units erythrocyte (BFU-E, CFU-E), CFU granulocyte-macrophage (CFU-GM) and CFU granulocyte-erythrocyte-monocyte-macrophage (CFU-GEMM) were assayed as described before.[18]

Analysis of homing receptors on allogeneic HSC

Mononuclear cells (MNC) from granulocyte-colony stimulating factor (G-CSF) mobilized allogeneic donors were isolated as described before.[18] Expression of homing receptors (CD44, CD184, CD49d, CD49e and $\alpha_9\beta_1$ integrin) was measured by flow cytometry. For further information see the *Online Supplementary Appendix.*

Immunohistochemistry

Immunohistochemistry (IHC) on BM sections was performed with the Histofine® Simple Stain MAX PO (Nichirei Biosciences INC, Tokyo, Japan) and DAB chromogen (ImmunoLogic, Duiven, The Netherlands) according to the manufacturer`s instructions. Expression of OPN, anti-intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, CD34 and SDF-1 was analyzed using an immunohistological score. More information is available in the *Online Supplementary Appendix*.

ELISA for soluble VCAM-1

Soluble (s)VCAM-1 was analyzed in the serum of MF, AML and healthy controls as described in the *Online Supplementary Appendix*.

Statistical analysis

Data are presented as median (range) and count (percentage). The probabilities of neutrophil and platelet engraftment, overall survival (OS) and non-relapse mortality (NRM) were calculated from date of transplant, according to the Kaplan-Meier product-limit method. NRM was defined as death without relapse. To determine factors affecting these end points, a log-rank test was performed and variables were significant at $P \leq 0.05$. Because of the small number of patients only univariable analyses were conducted. Flow cytometric data are presented as average + standard error of the mean (SEM). Student's *t*-test, Mann-Whitney U-test or ANOVA were used when appropriate for determining statistical significance. Analyses were performed using SPSS (IBM, Ehningen, Germany) or PRISM (Graphpad, San Diego, CA, USA) statistical software.

Results

Engraftment

The cumulative incidence of early and late neutrophil and platelet engraftment is shown in Figure 1. The mean time to early neutrophil engraftment in MF patients was 22 days (range 11-48) and in AML patients 17 days (range 10-23) (P=0.001) (Figure 1A). MF patients showed late neutrophil engraftment after a mean time of 35 days (range 13-65) and AML patients needed 25 days (range 12-55) (Figure 1B). Thirty percent of MF *versus* 7% of AML patients did not reach neutrophil engraftment by day 100 (P<0.001). The mean time to early platelet engraftment in MF patients was 31 days (range 9-92) and in AML patients 23 days (range 11-55) (P=0.094) (Figure 1C). MF patients showed late platelet engraftment after 33 days (range 11-96), whereas AML patients needed 27 days (range 12-73). Fifty-six percent of MF *versus* 23% of AML patients did not reach platelet engraftment by day 100 (P=0.01) (Figure 1D). GF only occurred in patients with MF. Primary GF was seen in 2 MF patients (6%) and secondary GF in 5 MF patients (16%) (Table 2). There was no significant difference in early and late neutrophil engraftment in MF patients with regard to donor type (Figure 2E and F). However, AML patients showed a significantly faster early (P<0.001) and late (P<0.01) neutrophil engraftment using MRD (Figure 2G and H). MF patients who had splenectomy before HSCT and MF patients with a spleen size smaller than the median of 21 cm (range 11-40 cm) showed a significantly faster early neutrophil engraftment (P=0.01 and P=0.03, respectively) (Figure 2A); however, spleen size or splenectomy had no impact on late neutrophil engraftment (Figure 2B). Regarding early platelet engraftment, there was no significant difference between splenectomy and spleen size smaller or larger than the median (Figure 2C), whereas for late platelet engraftment it was shown that patients after splenectomy had a significantly faster engraftment compared to patients with spleen size smaller (P=0.02) or larger than the median (P=0.03) (Figure 2D). For 19 MF

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patients, data on spleen size could be collected 2-4 months after transplantation and were reduced on average by 20.5% (5.4 cm, range 0.3-13 cm). Higher numbers of transplanted cells [> $6x10^6$ cells/kg/body weight (BW)], blood group (ABO) match status, age, GvHD, BM fibrosis grade, and number of blasts were not associated with neutrophil engraftment.

Survival, relapse and non-relapse mortality

With a follow up of 24 months after HSCT, 2-year OS was 54% (95%CI: 36-72) in MF patients and 58% (95%CI: 41-76) in AML patients (*P*>0.05) (Figure 3A). A total of 14 MF patients died. Causes of death were: graft failure (n=1), infection (n=6; including 2 patients with secondary GF), hemorrhage (n=1), GvHD (n=2), multi-organ failure (n=2) and relapse (n=1). In the AML group, 13 patients died due to relapse (n=6), infection (n=1), GvHD (n=3) and multi-organ failure (n=3). Two-year NRM is 42% (95%CI: 18-60) in MF and 23% (95%CI: 7-40) in AML patients, respectively.

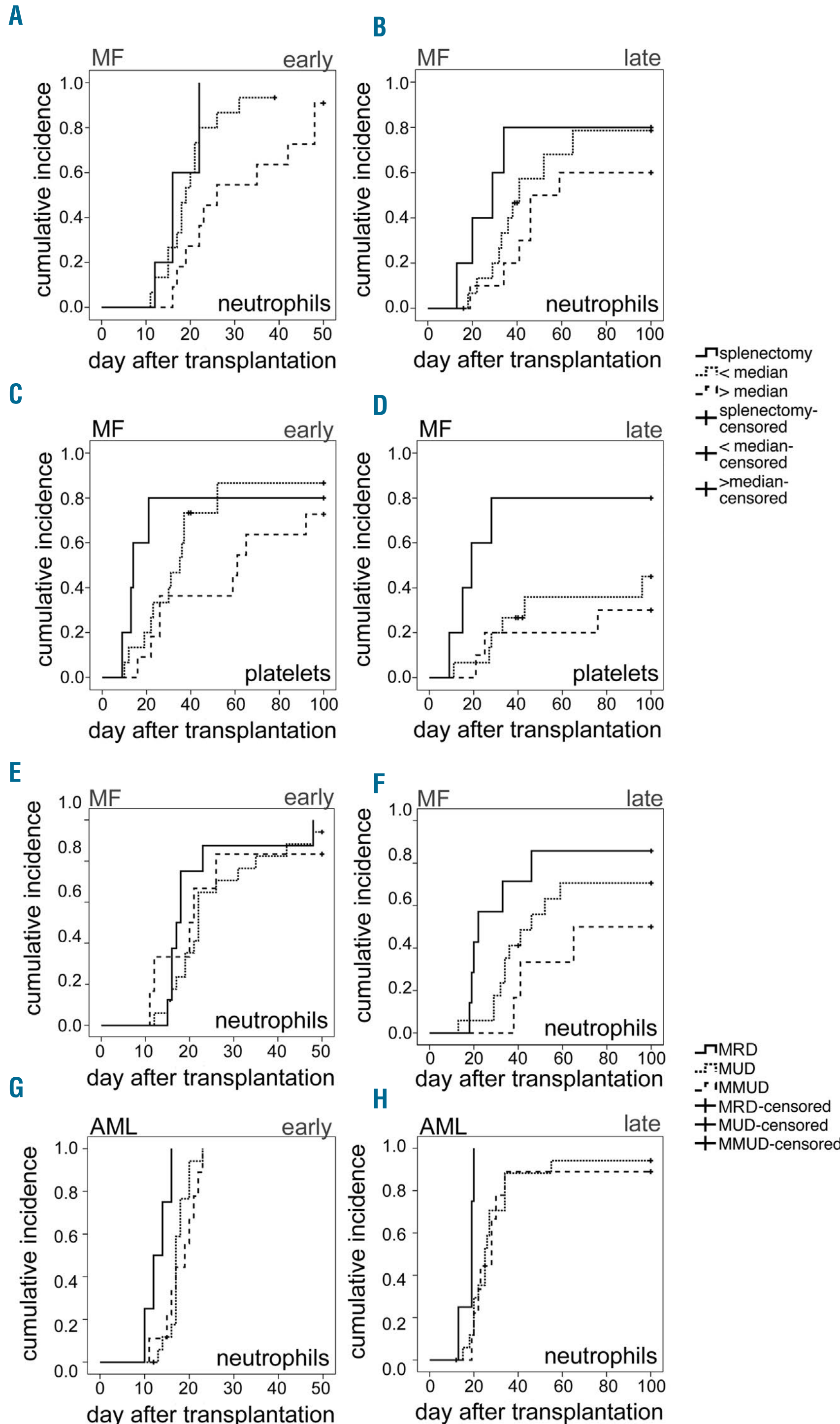

Figure 2. Spleen size and splenectomy but not donor type are associated with improved engraftment in myelofibrosis (MF) patients. Cumulative incidence of engraftment according to spleen and donor characteristics. Early (A) and late (B) neutrophil and early (C) and late (D) platelet engraftment in MF patients with regard to spleen size. Early and late neutrophil engraftment in MF (E and F) and acute myeloid leukemia (G and H) patients with regard to donor type. MRD: matched related donor; MUD: matched unrelated donor; MMUD: mismatched unrelated donor.

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Transfusions

The average number of erythrocyte transfusions after 28 and 100 days was significantly higher in MF patients than in AML patients (12.2±1.1 and 20.7±2.7 *vs.* 6.6±0.6 and 9.1±1.0, respectively; $P<0.05$) (Figure 3B). Accordingly, average numbers of platelet transfusion (Figure 3C) was significantly higher in patients with MF as compared to AML (11.5±1.3 and 18.8±3.2 *vs.* 7.7±0.8 and 10.3±1.4, respectively; $P<0.05$).

Clearance of HSC after HSCT

Our clinical data indicate that early and late neutrophil engraftment, as well as late but not early platelet engraftment, is significantly delayed in patients with MF as compared to AML. Therefore, we analyzed the number of circulating HSC within the PB by flow cytometry in 10 patients shortly before and after transplantation as a surrogate marker for the homing efficiency. Average age of these patients was 64.6±1.6 years (MF) and 50.0±1.8 years (AML). Spleen size was 18.7±3.1 cm (MF) and 12.2±2.0 cm (AML) ($P<0.05$). Figure 4A shows representative dot-plots of $CD34^+$ cells within the PB of one recipient. After 40 min, more than 75% of the transplanted $CD34^+$ cells were cleared from the PB (Figure 4B), indicating a high homing efficiency. However, significant differences in the number of circulating cells were seen between MF and AML. After 10, 20 and 40 min 1475±244, 1066±127 and 456±61 $CD34^+$ cells/mL blood circulate in AML as compared to 682±186, 489±162 and 306±81 cells in MF patients ($P<0.05$). Interestingly, the numbers equalize after 6 h and thereafter. At 22 h, 27±12 and 11±2 $CD34^+$ cells/mL and after 3-5 days 24±10 and 10±3 $CD34^+$ cells/mL were detectable for MF and AML patients, respectively. In addition, the number of $CD34^+CD38^-$ cells in the PB was analyzed. There was no significant difference in circulating $CD34^+CD38^-$ in MF compared to AML patients (*Online Supplementary Figure S1*). The proportion of CD38- cells within the $CD34^+$ cell population was significantly (approx. 3.5 times) higher up to 160 min in MF patients as compared to AML patients (Figure 4C).

Clearance of CFC

As the expression of CD34 and CD38 on HSC is of limited use with regard to the biological property of the cells, we compared the number of CFC between MF and AML patients at the same time points using a methylcellulose assay. (Figure 4D shows the representative example of white and red colonies; there is no significant difference in CFC numbers as shown in Figure 4E).

Expression of homing molecules on $CD34^+$ cells

To determine the expression of homing molecules on transplanted allogeneic PB-HSC, we analyzed the expression of crucial homing receptors, namely CD44, CD184, CD49d, CD49e and $\alpha_9\beta_1$ integrin before infusion. Figure 5A shows one representative flow analysis. Taken together, more than 99% of $CD34^+$ and $CD34^+CD38^-$ cells express CD44, CD49d and CD49e; 93.6±3% and 87.5±6% of the cells express $\alpha_9\beta_1$ and 40.4±6% and 40.2±10% express CXCR4 (CD184), respectively.

Immunohistochemistry of homing-related niche proteins

Since our clinical and experimental data suggest that the decreased long-term engraftment characteristics in MF compared to AML patients cannot be sufficiently explained solely by the spleen size, we analyzed the expression of homing-related proteins within the BM prior to conditioning chemotherapy. As expected, increased BM cellularity and increased numbers of vessels visualized by CD34 staining was detected in MF patients. Further immunohistochemical analyses revealed no significant difference with regard to SDF-1, OPN or ICAM-1 expression between MF and AML BM (*Online Supplementary Figure S2*). However, a significant loss of VCAM-1 expression in MF patients was detected (Figure 5B). To determine whether this VCAM-1 loss persists after transplantation, 11 MF patient samples were scored before and 5 samples after HSCT. There was a significantly lower VCAM-1 expression in MF patients before HSCT (median IRS=1) as compared to corresponding samples of MF patients after transplantation (median IRS=6; $P<0.05$) (Figure 5C). AML patients (n=4, median IRS=6; $P<0.05$) and healthy controls (n=6) showed VCAM-1 expression levels in BM similar to post-transplant MF patients. We also studied VCAM-1 expression in the bone marrow of PV (n=5) and ET (n=4) patients. IRS values ranged from 1 to 9 (PV) and 1 to 4 (ET), respectively, and thus were similar to levels observed in AML patients and healthy controls (*data not shown*).

Soluble VCAM-1 in serum of MF and AML patients

One possible explanation for the loss of VCAM-1 expression in MF patients is the cleavage by proteases

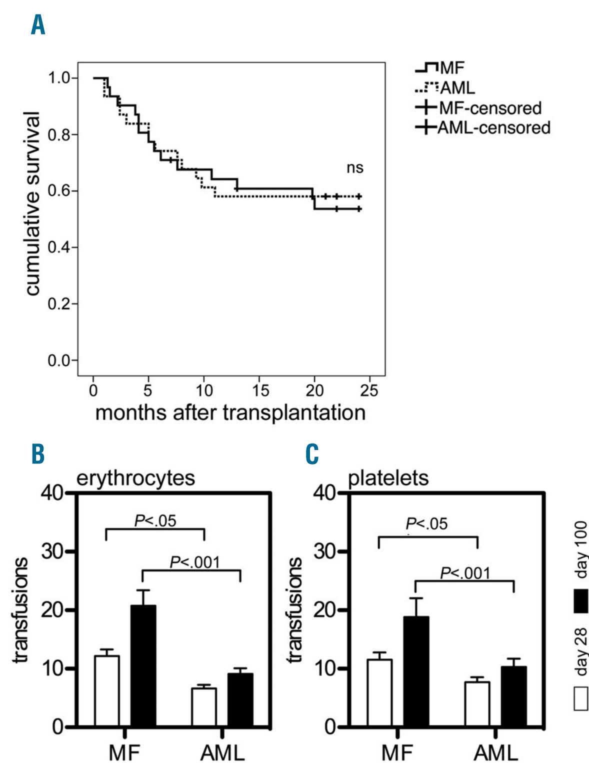

Figure 3. Overall survival is equal in myelofibrosis (MF) and acute myeloid leukemia (AML) patients but transfusion needs significantly differ. (A) Kaplan-Meier estimate of survival in MF and AML patients with a follow up of 24 months after allogeneic stem cell transplantation. Average number of erythrocyte (B) and platelet (C) transfusions in MF and AML patients on day 28 and 100 after allogeneic stem cell transplantation.

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within the modified MF BM. To test this hypothesis, the level of sVCAM-1 within the MF patient serum was analyzed in comparison to AML patients and healthy controls. sVCAM-1 level was significantly higher in the serum of MF patients (n=8, 1672±288 ng/mL) as compared to AML patients (n=3, 747±106 ng/mL) ($P<0.05$) and healthy controls (n=8, 595±56 ng/mL) ($P<0.05$), respectively (Figure 5D).

Discussion

This study presents retrospective data on neutrophil and platelet engraftment after allogeneic HSCT from 62 matched MF and AML patients. Our data indicate that MF patients show significantly delayed early and late neutrophil, as well as late platelet engraftment, compared to the AML cohort. Analysis of engraftment-related factors

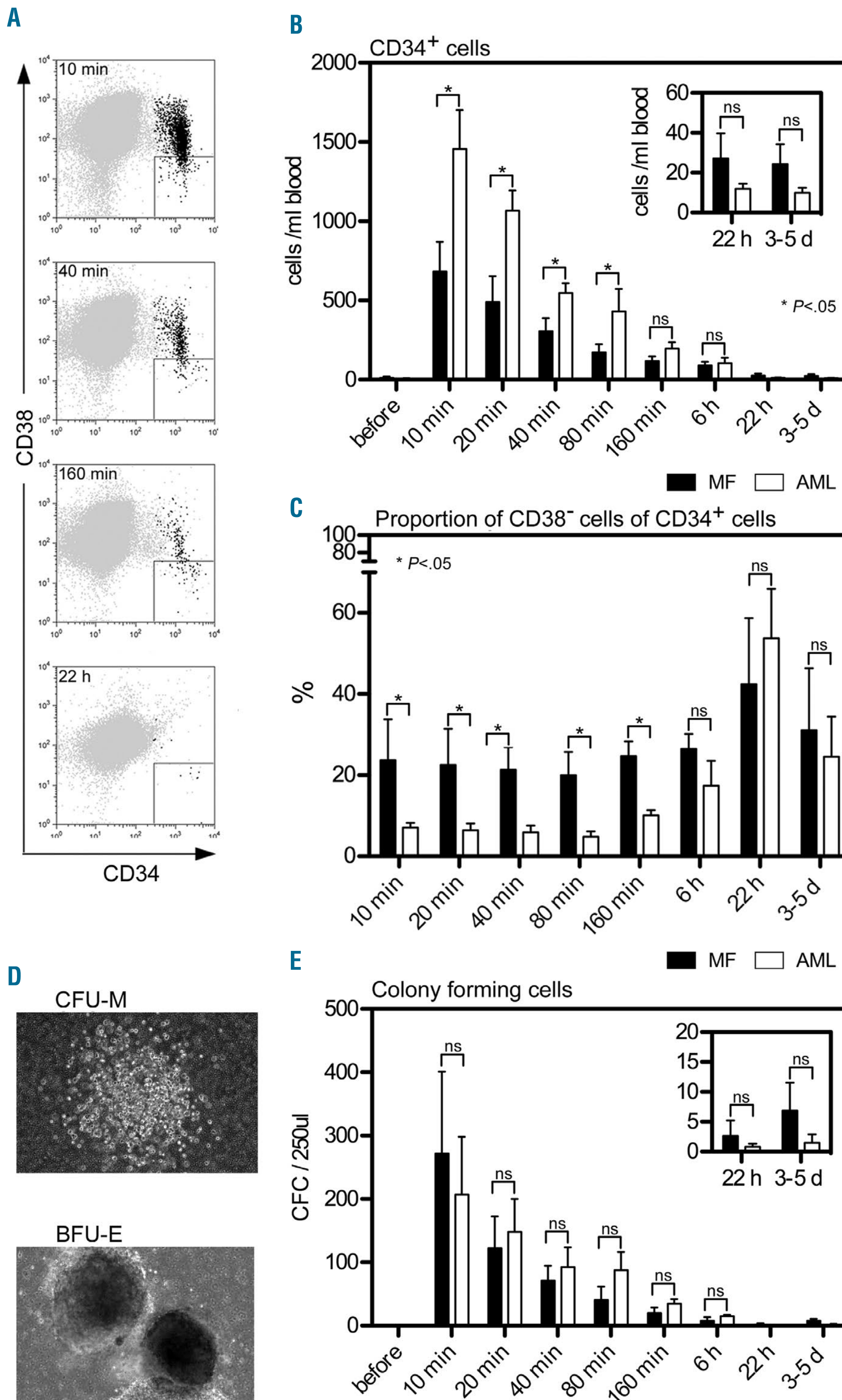

Figure 4. Clearance of CD34+ cells in myelofibrosis (MF) patients is significantly different to acute myeloid leukemia (AML) patients. Clearance of CD34+ cells after allogeneic stem cell transplantation in MF and AML patients at defined time points. (A) Representative dotplot of CD34 and CD38 stained HSC within the peripheral blood of one recipient. Clearance of CD34+ cells (B) and proportion of CD38- cells within the CD34+ cell fraction (C) in MF (n=5) and AML (n=5) patients. (D) Representative white (CFU-M) and red (BFU-E) colonies after hematopoietic stem cell transplantation. (E) Clearance of colony-forming cells (CFC) in MF and AML patients at defined time points after transplantation.

revealed no correlation to the blood group (ABO), age, GvHD, BM fibrosis grade, number of blasts prior to transplantation, or donor source. Interestingly, Robin *et al.*[8] and Rondelli *et al.*[19] reported a significant difference in engraftment between MRD and MUD. This divergence might be due to the somewhat smaller number of patients within our study. Interestingly, primary GF was seen in 2 MUD transplanted MF patients, whereas no difference with regard to donor source was detected in the rate of secondary GF.

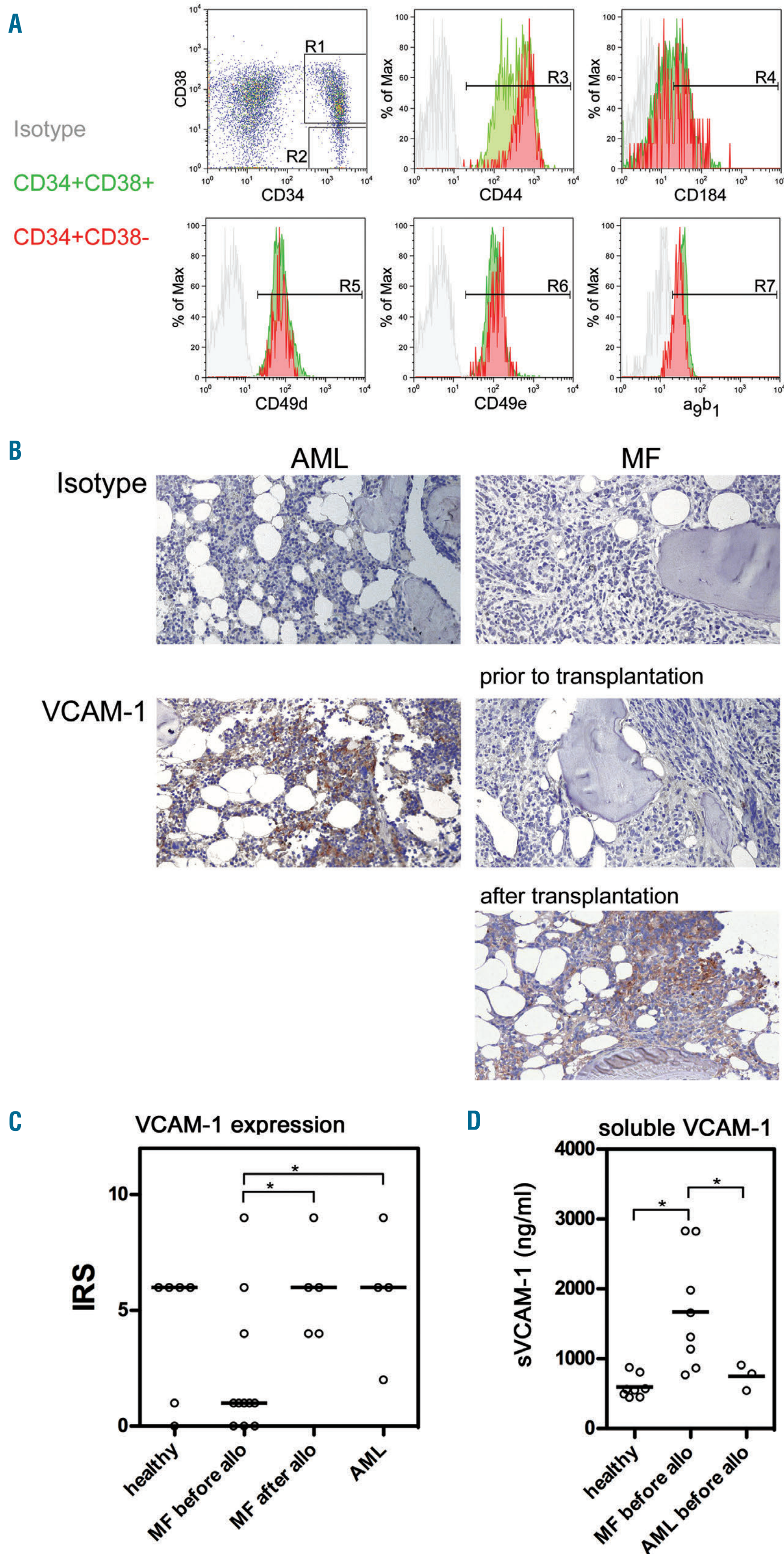

Figure 5. Peripheral blood (PB) hematopoietic stem cells (HSC) express common homing receptors but loss of VCAM-1 is detected in the bone marrow of myelofibrosis patients prior to hematopoietic stem cell transplantation (HSCT). (A) Flow cytometric analysis of the homing receptors CD44, CD184, CD49d, CD49e and $\alpha 9\beta 1$ integrin on $CD34^+CD38^+$ and $CD34^+CD38^-$ cells. (B) Representative immunohistology staining of VCAM-1 on acute myeloid leukemia (AML) and MF patients derived BM sections shortly before conditioning chemotherapy as compared to isotype control and one representative MF BM section eight months after HSCT. (C) Immunohistochemical rating score (IRS) for VCAM-1 expression in BM of MF patients (before HSCT n=11, after HSCT n=5), AML patients (n=4) and healthy controls (n=6). Each data point represents the individual VCAM-1 IRS and bar represents the median of sample groups. (D) Expression of soluble VCAM-1 in the serum of MF patients (n=8) as compared to AML patients (n=3) and healthy controls (n=8). Each data point represents the mean individual sVCAM-1 concentration assayed in duplicate and bar represents the mean of sample groups. *$P<0.05$

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Previous transplantation data suggest that the time to engraftment is dependent on the number of transplanted CD34+ cells. In the allogeneic setting 2.5-11.0x10^6 CD34+ cells/kg/BW are considered safe.[20] The median number of transplanted cells in our study was 5.81 (MF) and 5.98 (AML)x10^6 CD34+ cells per kg/BW, respectively, and did not correlate with the neutrophil engraftment.

The cumulative incidence of NRM at two years is significantly higher in MF than in AML patients and is slightly higher as previously reported by Claudiani *et al*.[21] after RIC. Fittingly, not only NRM, but also number of blood and platelet transfusions was significantly higher in MF patients implying that HSCT in these patients results in increased morbidity and costs. However, there was no significant difference in 2-year OS between MF and AML patients (54% and 58%, respectively).

As allogeneic HSCT provides the only curative treatment option for MF so far, we analyzed factors affecting the transplantation outcome. Effective homing of human HSC into the BM is a prerequisite for successful engraftment after transplantation. After attaching to adhesion proteins on BM vessels, the transplanted HSC trans-migrate through the endothelium and marrow and finally lodge into the stem cell niche. This process is highly regulated by a dynamic interaction of chemokines and adhesion molecules to ensure a purposive homing and engraftment.[22]

We recently demonstrated that more than 80% of transplanted murine HSC home to the BM within 5 h.[23] As our clinical data indicate that early engraftment of neutrophils is significantly delayed in MF patients, we used the clearance of the HSC from the PB as a surrogate marker for the homing of HSC. We show that more than 75% of the transplanted CD34+ cells exited the PB within 40 min and the number of circulating CD34+ cells after 22 h was the same as that prior to transplantation. A similar approach was used by Donmez *et al*.,[24] showing that nearly all autologous transplanted CD34+ cells exit the PB within 24 h.

Interestingly, the number of circulating CD34+ cells in MF patients was significantly lower at early time points (up to 80 min) compared to AML patients. This suggests that lineage specific committed CD34+ cells are initially pooled within the spleen of MF patients. This is supported by the fact that splenectomy before HSCT significantly accelerates early neutrophil engraftment. In this regard, the only factors favorably affecting engraftment in MF patients in our study were spleen size smaller than the median of 21 cm and splenectomy, as described before.[6,8,9,25] On the other hand, pooling of CD34+ cells within the spleen is a key feature of myelofibrosis.[26] Early animal studies proposed that neutrophil pooling strongly depends on the spleen size,[27] therefore, low neutrophil count in MF patients is possibly a combined phenomena including reduced engraftment of precursors and pooling of mature neutrophils.

In contrast, the CD34+CD38- HSC fraction that is enriched for primitive HSC[28] showed no significant clearance difference between AML and MF patients at any time point, but there was a trend towards a higher number of circulating cells in MF. This finding is congruent with early animal data demonstrating that primitive murine HSC display a preferential homing to the BM rather than to the spleen.[29] At later time points, the number of circulating HSC was higher in MF than in AML patients, yet this difference was not significant due to the very low numbers of cells within the PB measurable. It can be speculated that, firstly, short-term engrafting HSC (ST-HSC), that are responsible for early engraftment up to 8-12 weeks, preferentially home to the spleen in MF patients, being less supportive for early reconstitution than the BM. Secondly, long-term engrafting HSC (LT-HSC) show a prolonged circulation due to a homing defect eventually causing a reduced late engraftment. This is supported by the fact that spleen size or splenectomy had no impact on late neutrophil engraftment; however, we cannot prove this assumption as the ST- or LT-HSC phenotype[30] of the circulating cells was not analyzed.

To determine the cause for the engraftment defect we studied key homing molecules. One essential protein for HSC homing is SDF-1.[12,31] Disruption of SDF-1 binding to its receptor CXCR4 expressed on HSC and suppression of SDF-1 in osteoblasts after G-CSF administration leads to a sustained mobilization of HSC into the PB.[32,33] SDF-1 is upregulated in MF patient spleens, possibly explaining the preferential homing of ST-HSC to this extramedullary site.[34,35] On the other hand, Migliaccio *et al*. demonstrated in gata-1 deficient MF mice and also MF patients a higher SDF-1 expression within the BM.[36] Moreover, we recently demonstrated that OPN, expressed by osteoblasts within the endosteal niche, also has chemotactic activity.[37] However, immunohistology of BM sections obtained before transplantation did not show any significant difference in SDF-1 and OPN expression in AML and MF patients.

As adherence of circulating HSC to endothelial cells *via* VCAM-1 and ICAM-1[38] induces the homing process, we further analyzed the expression of these proteins. Whereas no difference in ICAM-1 expression was seen, there was a significant loss of VCAM-1 in BM samples from MF patients as compared to AML patients. After transplantation and reconstitution of BM, the VCAM-1 expression increased to normal levels. VCAM-1 is commonly expressed by BM stromal cells and endothelial cells,[39] and is a key protein for the adhesion of HSC to the endothelium before migration into the BM.[38] Cleavage of VCAM-1 by metalloproteases after application of G-CSF results in HSC mobilization.[40] In this context, data of Xu *et al*.[41] indicate that a proteolytic environment within the BM of MF patients results in the cleavage of VCAM-1 and increased plasma level of cleaved VCAM-1, which is in accordance with our findings. This leads to the constitutive mobilization of CD34+ cells into the PB. Therefore, one can suggest that the cleavage of VCAM-1 not only results in an increased mobilization of steady state recipient HSC in MF patients, but also causes a homing defect responsible for the reduced engraftment of LT-HSC.

As an engraftment defect not only results in increased risk of therapy-related mortality but also higher costs for blood products and antibiotics, approaches to accelerate reconstitution should be discussed. One possible method would be to improve HSC homing by reduction of spleen size and of the cleavage of VCAM-1 by reducing the proteolytic activity within the BM. Ruxolitinib, the only commercially available JAK-2 inhibitor approved as therapy for MF, leads to a modulation of BM microenvironment, reduces fibrosis, and has an anti-inflammatory action.[42-44] First results for patients pre-treated with the JAK-2 inhibitor before HSCT were presented by Jaekel *et al*.[45] These showed a significant reduction of spleen size and cytokine-induced clinical side effects. However, primary engraftment failure was seen in 7% of the patients, as compared to 6% in our study, and no data on time to engraftment was given. Thus, further studies evaluating other anti-inflammatory drugs are needed.

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