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

by Christina Hart, Sabine Klatt, Johann Barop, Gunnar Mueller, Roland Schelker, Ernst Holler, Elisabeth Huber, Wolfgang Herr, and Jochen Grassinger

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

Short title: Engraftment defect in patients with myelofibrosis

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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 was 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. This was mirrored by a significant increased 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.

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 therapy evaluation \(^3\).

Treatment options comprise conventional drugs including anti-proliferative medication, immunomodulatory drugs (iMiDs), Janus kinase (JAK) inhibitors and
hematopoietic growth factors. However, allogeneic hematopoietic stem cell (HSC) transplantation (T) is the only curative treatment that is recommended to transplant-eligible intermediate 2 and high-risk patients.\textsuperscript{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).\textsuperscript{5,6} However, graft failure (GF) up to 10% in MF patients after RIC HSCT is a critical contributor to morbidity and mortality.\textsuperscript{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.\textsuperscript{8,9} The reconstitution of the BM after transplantation depends on the successful homing of transplanted HSC.\textsuperscript{10} After leaving the peripheral blood (PB) the HSC lodge into the stem cell niche that was shown to regulate the HSC pool.\textsuperscript{11} Several cellular components were identified to regulate the hematopoietic homeostasis, among them endothelial cells, mesenchymal stroma cells (MSC) and osteoblasts.\textsuperscript{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.\textsuperscript{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.\textsuperscript{15,16} Therefore, it can be speculated that the disarrangement of the BM niche in MF is one aspect for GF.\textsuperscript{17}

In this study we investigated neutrophil and platelet engraftment in patients with MF and acute myeloid leukemia (AML) following RIC-HSCT. Additionally, 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

Patient, 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. Patient and disease characteristics as well as donor and transplant procedures are summarized in table 1. The study was approved by the local ethics committee (number 02/220). For further information see Online Supplementary Data.

Clearance of HSC and colony-forming cells (CFC) from the PB after HSCT
Clearance of CD34+, CD34+CD38- cells and 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.

Flow cytometric analysis of circulating HSC
For analysis of the CD34+ and CD34+CD38- cell count, PB was lysed in NH₄CL lysis buffer and cells were then stained for 30 minutes 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, US). Detailed information is provided in Online Supplementary Data.

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 before18.

Analysis of homing receptors on allogeneic HSC
Mononuclear cells (MNC) from granulocyte-colony stimulating factor (G-CSF) mobilized allogeneic donors were isolated as described before18. Expression of homing receptors (CD44, CD184, CD49d, CD49e and α9β1 integrin) was measured by flow cytometry. For further information see Online Supplementary Data.
**Immunohistochemistry (IHC)**

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 instruction. 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 Data*.

**ELISA for soluble VCAM-1**

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

**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 endpoints, a log-rank test was performed and variables were significant at a *p*-value ≤ .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* was used when appropriate for determining statistical significance. Analyses were performed using SPSS (IBM, Ehningen, Germany) or PRISM (Graphpad, San Diego, CA, US) 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) ($p = .001$, 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) as shown in figure 1B. 30% of MF vs. 7% of AML patients did not reach neutrophil engraftment by day 100 ($p < .001$). 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) ($p = .094$, figure 1C). MF patients showed late platelet engraftment after 33 days (range 11-96), whereas AML patients needed 27 days (range 12-73). 56% of MF vs. 23% of AML patients did not reach platelet engraftment by day 100 ($p = .01$, figure 1D). GF only occurred in patients with MF. As shown in table 2, primary GF was seen in two MF patients (6%) and secondary GF in five MF patients (16%).

As illustrated in figure 2, there was no significant difference in early and late neutrophil engraftment in MF patients in regard to donor type (figures 2E and F). However, AML patients showed a significantly faster early ($p < .001$) and late ($p < .01$) neutrophil engraftment using MRD (figures 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 ($p = .01$ and $p = .03$, 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 ($p = .02$) or larger than the median ($p = .03$, figure 2D). 19 MF patients data on spleen size could be collected 2-4 months after transplantation and was in average 20.5% reduced (5.4 cm, range 0.3-13 cm). Higher numbers of transplanted cells ($> 6 \times 10^6$ cells/kg/bw) and blood group (ABO) match status, age, GvHD, BM fibrosis grade and number of blasts was 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 (figure 3A, p > .05). A total of 14 MF patients died. Causes of death were: graft failure (n = 1), infection (n = 6; including two 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). The NRM at 2 years is 42% (95% CI, 18-60) in MF and 23% (95% CI, 7-40) in AML patients, respectively.

**Transfusions**

As shown in figure 3B, 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 < .05). 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 < .05).

**Clearance of HSC cells 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 < .05). Figure 4A shows representative dotplots of CD34+ cells within the PB of one recipient. After 40 min > 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 minutes 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 < .05). Interestingly, the numbers equalize after 6 hours and thereafter. At 22 hours 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.
Additionally, 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 (data is included in the supplement, figure S1). Therefore, as shown in figure 4C, the proportion of CD38⁻ cells within the CD34⁺ cell population was significantly (approximately 3.5 times) higher up to 160 min in MF patients as compared to AML patients.

**Clearance of CFC**

As the expression of CD34 and CD38 on HSC is of limited use in 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 one representative example of white and red colonies. As indicated in figure 4E, there is no significant difference in CFC numbers.

**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 α9β1 integrin before infusion. Figure 5A shows one representative flow analysis. Taken together, > 99% of CD34⁺ and CD34⁺CD38⁻ cells express CD44, CD49d and CD49e. 93.6 ± 3% and 87.5 ± 6% of the cells express α9β1 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 sufficiently be 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 in regard to SDF-1, OPN or ICAM-1 expression between MF and AML BM (data is shown in the supplement, 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 five samples after HSCT. As shown in figure 5C, 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, \( p < .05 \)). AML patients (median IRS = 6, \( p < .05 \), \( n = 4 \)) 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 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. As seen in figure 5D, sVCAM-1 level was significantly higher in the serum of MF patients \( (1672 \pm 288 \text{ ng/ml, } \( n = 8 \)) \) as compared to AML patients \( (747 \pm 106 \text{ ng/ml, } p < .05, \ n = 3 \) ) and healthy controls \( (595 \pm 56 \text{ ng/ml, } p < .05, \ n = 8 \) ), respectively.

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

Previous transplantation data suggest that the time to engraftment is dependent on the number of transplanted CD34\(^+\) cells. In the allogeneic setting \( 2.5-11.0 \times 10^6 \) 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) \( \times 10^6 \) 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. 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 transmigrate 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.

Recently, we could demonstrate that > 80% of transplanted murine HSC home to the BM within 5 hours. 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 minutes and the number of circulating CD34+ cells after 22 hours was at the same level as prior to transplantation. A similar approach was used by Donmez et al. showing that nearly all autologous transplanted CD34+ cells exit the PB within 24 hours.

Interestingly, the number of circulating CD34+ cells in MF patients was significantly lower at early time points up to 80 minutes 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. On the other hand, pooling of CD34+ cells within the spleen is a key feature of myelofibrosis. Early animal studies proposed that neutrophil pooling strongly depends on the spleen size, therefore low neutrophil count in MF patients is possible 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 showed no significant clearance difference between AML and MF patients at any time point but 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. At later time points the number of circulating HSC was higher in MF than in AML patients, yet 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 proof this assumption as the ST- or LT-HSC phenotype 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. 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. SDF-1 is up-regulated in MF patient spleens possibly explaining the preferential homing of ST-HSC to this extramedullary site. 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. Moreover, we recently demonstrated that OPN, expressed by osteoblasts within the endosteal niche, also has chemotactic activity. 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 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 and is a key protein for the adhesion of HSC to the endothelium before migration into the BM. Cleavage of VCAM-1 by metalloproteases after application of G-CSF results in HSC mobilization. In this context, data of Xu et al. 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 \(^1\). 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 acts anti-inflammatory \(^{42-44}\). First results for patients pretreated with the JAK-2 inhibitor before HSCT were presented by Jaekel et al. showing a significant reduction of spleen size and cytokine induced clinical side effects \(^{45}\). 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 have to be conducted.

**Authorship**

C. Hart, S. Klatt and J. Grassinger planned and performed experiments and wrote the manuscript. J. Barop, G. Müller, E. Huber and R. Schelker performed experiments. E. Holler and W. Herr provided patient material and reviewed the manuscript. The authors declare no competing financial interests.
Bibliography


Table 1: Patient characteristics

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<th>Characteristics</th>
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<th>AML patients (n=31)</th>
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<td><strong>Donor, no (%)</strong> (on the basis of high HLA resolution testing (HLA-A, -B, -C, -DRB1 and -DQB1))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Related</td>
<td>8 (26)</td>
<td>Related 4 (13)</td>
</tr>
<tr>
<td>Unrelated matched</td>
<td>16 (52)</td>
<td>Unrelated matched 18 (58)</td>
</tr>
<tr>
<td>Unrelated mismatch</td>
<td>7 (22)</td>
<td>Unrelated mismatched 9 (29)</td>
</tr>
<tr>
<td><strong>Major ABO mismatch, no (%)</strong></td>
<td>11 (35)</td>
<td>Major ABO mismatch, no (%) 8 (26)</td>
</tr>
<tr>
<td><strong>GvHD prophylaxis, no (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSA + MTX</td>
<td>18 (58)</td>
<td>CSA + MTX 20 (65)</td>
</tr>
<tr>
<td>CSA + MMF</td>
<td>10 (32)</td>
<td>CSA + MMF 10 (32)</td>
</tr>
<tr>
<td>CSA alone</td>
<td>3 (10)</td>
<td>Everolimus 1 (3)</td>
</tr>
</tbody>
</table>

*JAK2V617F mutation was routinely tested after 2006*

** No pretreatment with JAK2 inhibitor (JAK2 inhibitor was not available at this time)
Table 2: Characteristics of the MF patients with primary or secondary graft failure

<table>
<thead>
<tr>
<th>Primary graft failure</th>
<th>Conditioning</th>
<th>Type of donor</th>
<th>ABO Blood group</th>
<th>Spleen size (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient # 1</td>
<td>RIC</td>
<td>MMUR</td>
<td>Minor mismatch</td>
<td>11</td>
</tr>
<tr>
<td>Patient # 2</td>
<td>RIC</td>
<td>MUR</td>
<td>Ident</td>
<td>&gt; 20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary graft failure</th>
<th>Conditioning</th>
<th>Type of donor</th>
<th>ABO Blood group</th>
<th>Spleen size (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient # 1</td>
<td>MAC</td>
<td>MUR</td>
<td>Major mismatch</td>
<td>26</td>
</tr>
<tr>
<td>Patient # 2</td>
<td>RIC</td>
<td>MUR</td>
<td>Major mismatch</td>
<td>40</td>
</tr>
<tr>
<td>Patient # 3</td>
<td>RIC</td>
<td>MRD</td>
<td>Ident</td>
<td>28</td>
</tr>
<tr>
<td>Patient # 4</td>
<td>RIC</td>
<td>MUR</td>
<td>Major mismatch</td>
<td>20</td>
</tr>
<tr>
<td>Patient # 5</td>
<td>RIC</td>
<td>MRD</td>
<td>Major mismatch</td>
<td>- (splenectomy)</td>
</tr>
</tbody>
</table>

Abbreviations: MAC = myeloablative conditioning; RIC = reduced-intensity conditioning; MRD = matched related donor; MUR = matched unrelated donor; MMUR = mismatched unrelated donor.
Figure Legends

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

Figure 2: Spleen size and splenectomy but not donor type is associated with improved engraftment in 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 in regard to spleen size. Early and late neutrophil engraftment in MF (E,F) and AML (G,H) patients in regard to donor type. Abbreviations: MRD = matched related donor; MUD = matched unrelated donor; MMUD = mismatched unrelated donor.

Figure 3: Overall survival is equal in MF and 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.

Figure 4: Clearance of CD34+ cells in MF patients is significantly different as compared to 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 PB 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 HSCT. (E) Clearance of colony-forming cells (CFC) in MF and AML patients at defined time points after transplantation.

Figure 5. PB HSC express common homing receptors but loss of VCAM-1 is detected in the BM of MF patients prior to HSCT.
(A) Flow cytometric analysis of the homing receptors CD44, CD184, CD49d, CD49e and α9β1 integrin on CD34+CD38+ and CD34+CD38− cells. (B) Representative immunohistology staining of VCAM-1 on AML and MF patients derived BM sections shortly before conditioning chemotherapy as compared to isotype control and one representative MF BM section 8 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 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 < .05
Supplementary methods

Methods

Patient, disease and transplantation characteristics
Spleen size was evaluated by ultrasonography to determine median longitudinal diameter. BM biopsy specimens were obtained during routine controls in each patient prior transplantation and BM fibrosis was graded according to the Bauermeister classification\(^1\). The conditioning regimens were chosen according to age, comorbidities and time of transplantation.

Definitions
The day of graft infusion was defined as day 0. The day of early and late neutrophil engraftment was defined as the first of three consecutive days with an absolute neutrophil count of \(> 0.5 \times 10^9/L\) up to day 50 and neutrophil counts \(> 2 \times 10^9/L\) up to day 100 after transplant, respectively. The day of early and late platelet engraftment was defined as the first of the seven consecutive days up to day 100 with platelet count \(> 20 \times 10^9/L\) and \(> 50 \times 10^9/L\) without transfusion, respectively. GF was analyzed within 12 months after HSCT. Primary GF was diagnosed when the patient never recovered from neutropenia with neutrophil count less than \(0.5 \times 10^9/L\). Secondary GF was defined as the complete loss of donor cells after initial engraftment. Relapse was defined as the reappearance of host cells and morphological criteria of MF and AML after remission. Graft-versus-host disease (GvHD) was graded according to standard criteria\(^2,3\).

Flow cytometric analysis of circulating HSC
The following monoclonal mouse anti-human antibodies were used: CD45-FITC (clone HI30, BD Pharmingen, Franklin Lakes, NJ, USA), CD34-APC (clone 581, Biolegend, San Diego, CA, USA) and CD38-PE (clone HIT2, BioLegend). CD34\(^+\) cell numbers were determined according to ISHAGE guidelines\(^4\). Additionally, CD34\(^+\)CD38\(^-\) cells were gated excluding the corresponding isotype control (< 98%). The number of circulating cells per ml blood was calculated by correcting the measured events for the number of transplanted cells, blood volume of the recipient and the duration of the infusion. Blood volume \((v)\) was calculated for female recipients \(v = \text{(body weight (kg) × 0.047 + 0.86)}\) and male recipients
\[ v = (\text{body weight (kg)} \times 0.041) + 1.53 \] according to \(^5\). Correction factors for transplanted cells (\(n\)), blood volume (\(b\nu\)) and duration (\(t\)) was calculated as follows:

\[ n = \left( \frac{\text{mean of pooled cells numbers}}{\text{cell number per kg body weight}} \right), \]

\[ b\nu = \left( \frac{v}{\text{mean of pooled } v} \right), \]

\[ t = \left( \frac{\text{time of transplantation (minutes)}}{\text{mean of pooled times}} \right) \]

The number of CD34\(^+\) cells or CD34\(^+\)CD38\(^-\) cells per ml blood (\(c\)) was then calculated using this formula: \(c = n \times b\nu \times t\).

**Analysis of homing receptors on allogeneic HSC**

Expression of homing receptors was measured by flow cytometry using anti-CD38-PerCP (clone HIT2, BioLegend), anti-CD184-PE (clone 12G5, BioLegend), anti-CD49d-PE (clone 9F10, BD Pharmingen), anti-\(\alpha_9\beta_1\) (clone Y9A2, Merck-Millipore, Darmstadt, Germany) and anti-CD44-PE (clone 515, BD Pharmingen) monoclonal antibodies (mAbs).

**Immunohistochemistry (IHC)**

The IHC was performed with the Histofine® Simple Stain MAX PO [(M) and (R)] (Nichirei Biosciences INC, Tokyo, Japan) according to the manufacturer’s instructions. In short, 3 \(\mu\)m sections from formalin-fixed paraffin-embedded BM specimens were placed on slides (Superfrost, Thermo Scientific, Walthamn, MA, US). Sections were dewaxed and antigen retrieval was done by heating in 10 mM citrate puffer (pH 6.0). Endogenous peroxidase was quenched by a 3% solution of hydrogen peroxide. Sections were then stained with anti-OPN (clone 53, Abcam, Cambridge, UK), anti-intercellular adhesion molecule (ICAM)-1 (CD54, clone H4, Santa Cruz Biotechnology, Dallas, TX, US), anti-VCAM-1 (CD106, clone E-10, Santa Cruz), anti-CD34 (clone QBEnd-10, Dako) and anti-SDF-1 (polyclonal, Santa Cruz) as well as an anti-mouse (Dako, Glostrup, Denmark) and anti-rabbit isotype antibody (Abcam, Cambridge, UK). Incubation was performed over night at 4 °C and all antibodies were used at a concentration of 2.0 \(\mu\)g/ml, except SDF-1 (1 \(\mu\)g/ml).

Visualization was made using the universal immuno-peroxidase polymer MAX-PO and Bright-DAB (ImmunoLogic, Duiven, The Netherlands) and sections were counterstained with Mayer’s hemalum solution (Merck KGaA, Darmstadt, Germany). Slides were scanned and analyzed using Zeiss Mirax Scan (Carl Zeiss GmbH, Göttingen, Germany) or images were obtained with a Zeiss Axioskop 2 Plus.
microscope with an AxioCam HRc camera and Axiovision 4.7.2 software (Carl Zeiss GmbH, Göttingen, Germany). No further image processing was performed despite adjustment of brightness and contrast. Staining for OPN, ICAM-1, SDF-1 and CD34 was performed with bone marrow samples of 3 MF patients, 3 AML patients and 3 healthy controls. VCAM-1 staining was conducted using BM sections of MF patients before allogeneic HSCT (n = 11) and 3-13 months after HSCT/BM reconstitution (n = 5) as well as of AML patients (n = 4), PV patients (n = 5), ET patients (n = 4) and healthy controls (n = 6). The expression of VCAM-1 was graded using an immunohistochemical rating score (IRS) according to Remmele and Stegner. In brief, the intensity was rated as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The amount of positive stained cells was rated as follows: 0, no cells; 1, < 10%; 2, 10–50%; 3, 51–80%; and 4, > 80%. IRS was calculated by multiplying scores of intensity and amount of positive cells. The specificity of immunoreactive signals for VCAM-1 was verified by negative controls using the above mentioned isotype control antibodies.

**ELISA for soluble VCAM-1**

Soluble (s)VCAM-1 was analyzed in the serum of MF, AML and healthy controls. Frozen serum samples of MF patients (n = 8) and AML patients (n = 3) before HSCT as well as samples of healthy controls (n = 8) were thawed and analyzed using the commercially available sVCAM-1 enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, US). All samples were the same as for IHC except the healthy controls. Serum was diluted 20-fold and assayed in duplicate in a 96-well plate according to the manufacturer’s instructions. sVCAM-1 measurement was performed by reading the wavelength at 450 nm with a correction wavelength set to 570 nm using the Tecan Sunrise microplate absorbance reader and the Magellan 5 data analysis software (Tecan Group Ltd., Switzerland). The sensitivity of that assay is 1.26 ng/ml as provided by the manufacturer.
Supplementary results

Figure S1

Clearance of CD34⁺CD38⁻ cells from the peripheral blood of MF and AML patients after HSCT. Figure shows the mean from 5 patients each.

Error bar = SEM
Figure S2

Representative examples of the expression of CD34, SDF-1 and OPN within the BM of MF and AML patients prior to HSCT
References