SUPPLEMENTARY APPENDIX

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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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 x 10^9/L up to day 50 and neutrophil counts > 2 x 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 x 10^9/L and > 50 x 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 x 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 $v = (body\ weight\ (kg) \times 0.047 + 0.86)$ and male recipients

$v = (body\ weight\ (kg) \times 0.041) + 1.53)$ according to [5]. Correction factors for transplanted cells (n), blood volume (bv) and duration (t) was calculated as follows:

$n = ((mean\ of\ pooled\ cells\ numbers\ ) \div (cell\ number\ per\ kg\ body\ weight))$,

$bv = ((v\ ) \div (mean\ of\ pooled\ v))$,

$t = ((time\ of\ transplantation\ (minutes)) \div (mean\ of\ pooled\ times))$

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

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 μm 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 μg/ml, except SDF-1 (1 μg/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

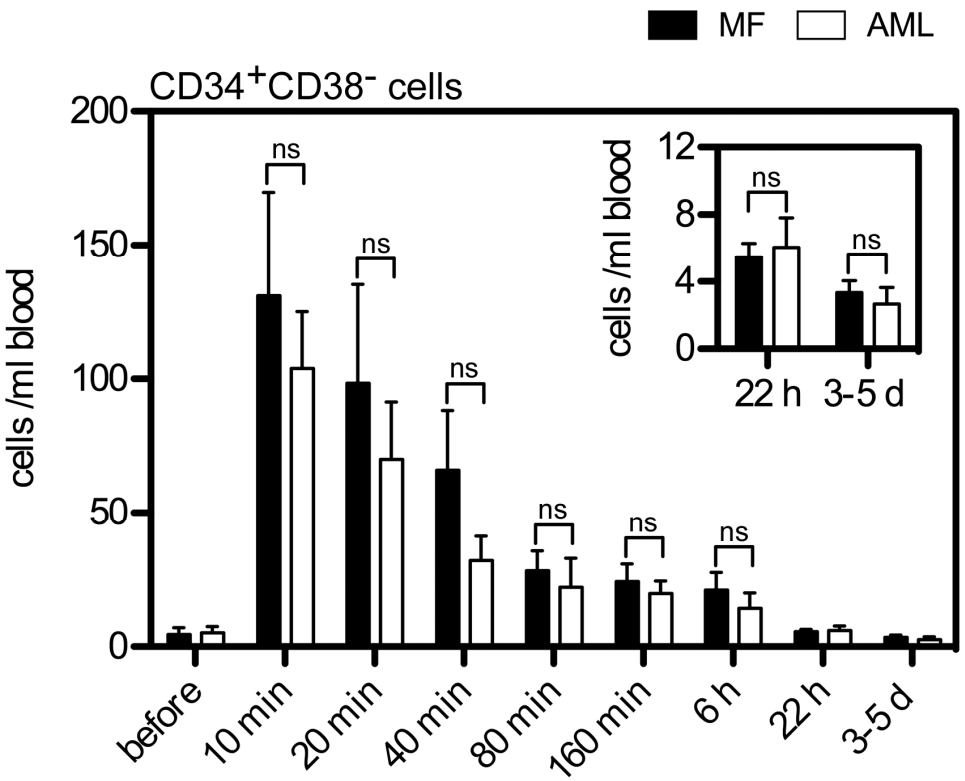
microscope with an AxioCam HRc camera and Axiovision 4.7.2 software (Carl Zeiss GmbH, Göttingen, Germany). No further image processing was performed despite adjustment of brightness and contrast. Staining for OPN, ICAM-1, SDF-1 and CD34 was performed with bone marrow samples of 3 MF patients, 3 AML patients 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 [6]. 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.

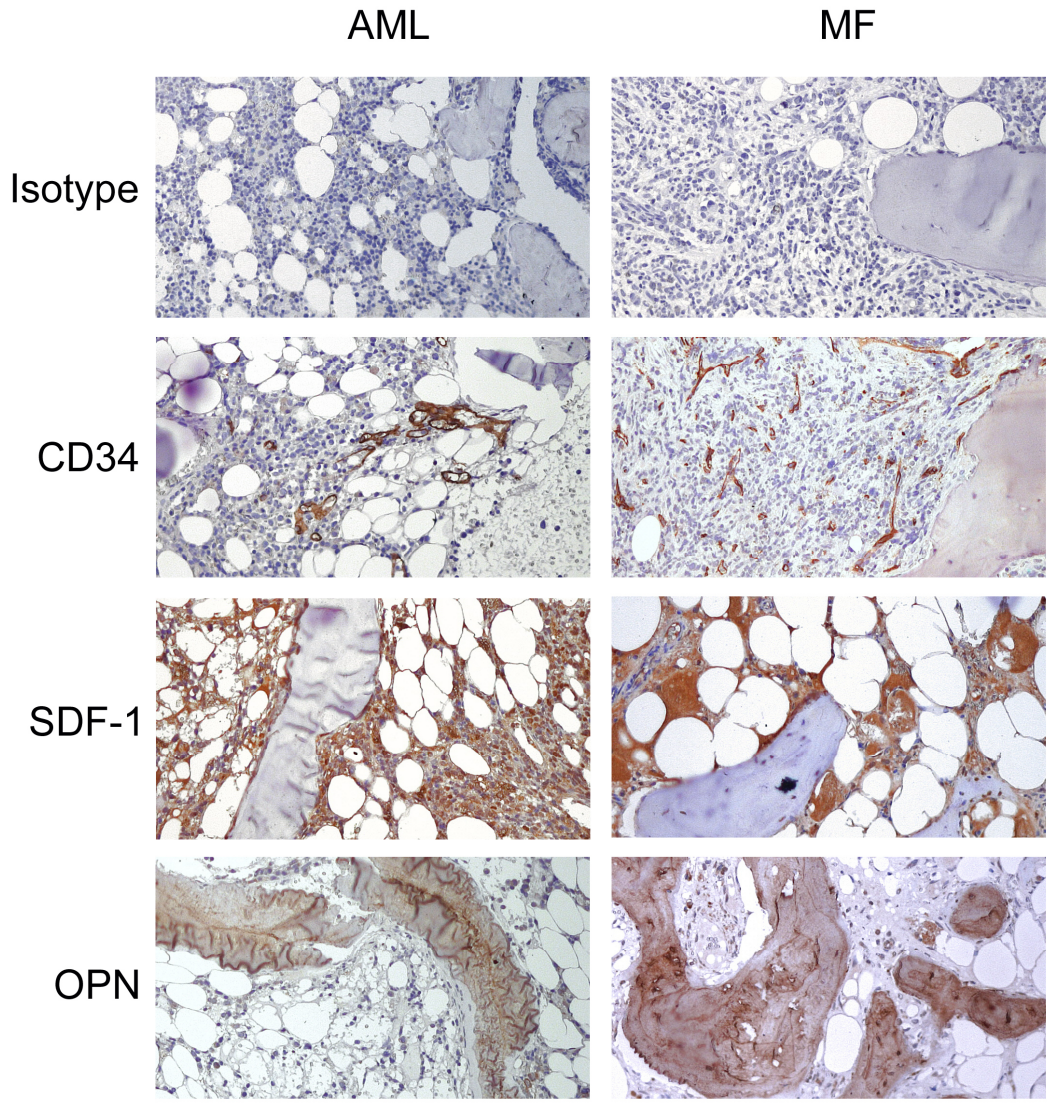
Supplementary results

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

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