Circulating endothelial cell enumeration demonstrates prolonged endothelial damage in recipients of myeloablative allogeneic stem cell transplantation

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SUPPLEMENTARY METHODS

Patients and blood collection

The institutional review board approved the protocols, and all patients and donors provided written informed consent. Peripheral blood (PB) samples were acquired in EDTA tubes at baseline (one month before transplantation) and 3, 6, 12 and 24 months post-transplant to determine post-transplant kinetics of CECs. In patients undergoing a double umbilical cord blood transplantation (dUCBT), additional PB samples for the same purpose were acquired at 1 and 2 months post-transplant. Samples were maintained at room temperature and processed within 24 hours of blood collection.

All MUD and sib donors received granulocyte colony-stimulating factor (G-CSF; 2x 5 µg/kg s.c.) to mobilize peripheral blood stem cells, starting at day -5 and ending at the last day of apheresis. Stem cells were infused at day 0 in all cohorts. In the dUCBT cohort, grafts were routinely infused at two consecutive days (day 0 and day +1). Hematopoietic growth factors (G-CSF) were not routinely given to allo-SCT recipients in any of the cohorts.

All patients received cyclosporine A (CsA; trough level 250-350 µg/l) and mycophenolate mofetil (MMF; 2 x 16 mg/kg) as additional post-transplant GVHD prophylaxis for at least three months and one month, respectively, with gradual tapering of the drug thereafter. Acute GVHD (aGVHD) was graded according to the Glucksberg criteria updated according to Przepiorka et al. (1, 2). All patients who suffered from aGVHD grade II-IV received prednisone (2 mg/kg/day). Chronic GVHD (cGVHD) was scored according to the Seattle classification for limited and extensive chronic GVHD (3). Chronic GVHD for which local therapy was not applicable, was treated with a combination of prednisone and cyclosporine according to clinical response.

All patients received prophylactic cotrimoxazol (1 x 480 mg) to prevent infections with pneumocystis carinii and valaciclovir (3 x 500 mg) to prevent CMV-reactivations for at least one year following allo-SCT. In the case of chronic GVHD or delayed immunosuppressive tapering, infectious prophylaxis was prolonged.

Infections

All infections were scored according to the NCI common toxicity criteria (CTC) version 3.0 (4) between day 1 and day 365 post-transplant, as described before (5, 6). All CTC grade 3-4 infections were scored and, if applicable, the location and causative microorganism of the infection were documented. In addition, CTC grade 2 CMV reactivations were scored, because CMV is known to infect endothelial cells and promote angiogenesis.

Enumeration of circulating endothelial cells

Enumeration of circulating endothelial cells (CECs) was performed according to our previously reported flow cytometric approach [23]. We used the following directly conjugated monoclonal antibodies for the identification of CEC: CD34-FITC (clone 8G12; BD Biosciences, San Jose, CA, USA), CD146-APC (clone 541-10B2; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and CD45-PerCP (clone 2D1; BD Biosciences). DRAQ5 (Biostatus Ltd, Shepshed, UK) was used as a cell permeable nuclear dye to exclude platelets and microparticles. CECs were defined as CD34+, CD146+, CD45- and DRAQ5+.

To study expression of HLA-DR on CECs, HLA-DR-PE (clone L243, BD Biosciences) was used. For the HLA-class I and HLA-mismatch analyses, HLA-A2, HLA-A9, HLA-B12, HLA-B27 & HLA-Bw6 biotinylated monoclonal antibodies (IgG2b; One Lambda, Canoga Park, CA, USA) were used and subsequently coupled to Streptavidin-PE (BD Biosciences).

Samples were acquired on a FACS Fortessa flow cytometer (BD Biosciences) and were subsequently analyzed using FCS Express (De Novo Software, Los Angeles, CA, USA). Analyses were always checked by one experienced technician to minimize inter-rater variability.

Statistical considerations

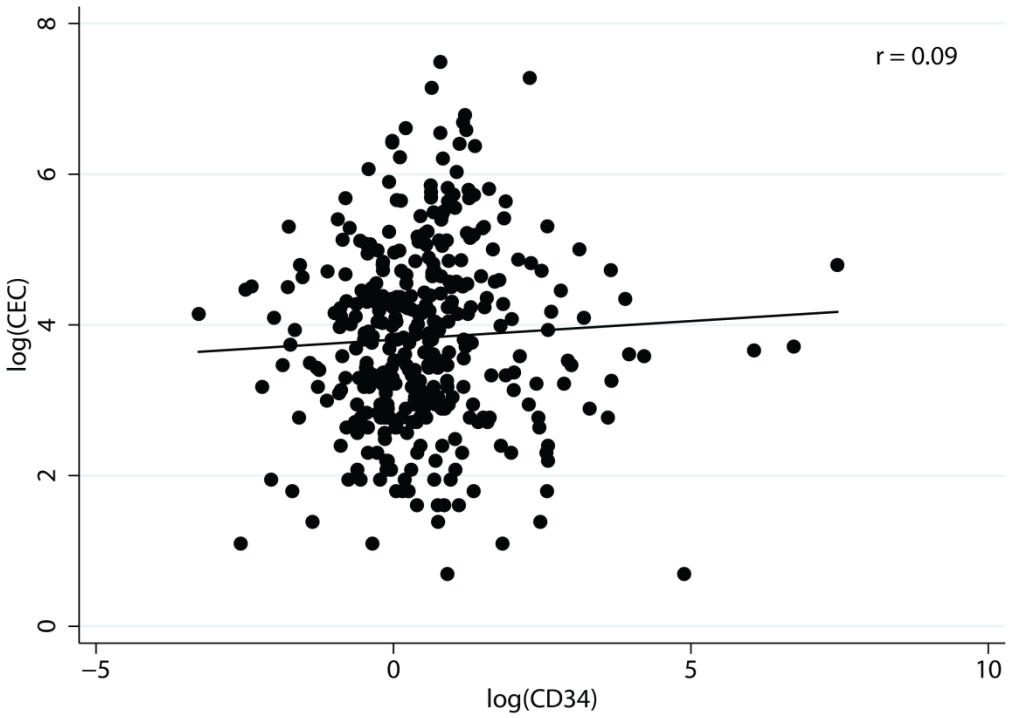
Several time intervals were constructed to define which CEC measurements were eligible for a given time point. A sample was considered a 'pre' sample if it was acquired at day 0 or -1, 1 month was defined as acquired between day +15 and +45, 2 months between day +46 and +75, 3 months between day +76 and +105, 6 months between day +155 and +205, 12 months between day +340 and +390 and 24 months between + 700 and +760. These time intervals were also used to define the presence of absence of GVHD at that given time point. CEC samples that were drawn after disease relapse were excluded from the analysis, as the presence of very high numbers of disease-related CD34+ stem cells in these patients may interfere with the CEC analysis. CEC numbers between conditioning types were compared using the Mann-Whitney U test. For the comparison of CEC numbers within the same patients on different time points, the Wilcoxon signed-rank test was used. Multivariable linear regression was performed using log-normalized CEC numbers to assure normality of the CEC data. Parameters used as variables included age, gender, HCT-CI score, donor source, conditioning regimen (MAB, RIC and UCB conditioning), occurrence of GVHD in the same interval as the CEC measurement and occurrence of infections in the 3 months prior to the CEC measurement. A backward stepwise approach was used with a significance level of ≥0.2 to omit a given variable from the model. Age and gender were then subsequently added to the model, even if they did not have a significant contribution to the model, to assure that the most clinically relevant model was used. All reported *p* values are two-sided unless stated otherwise, and a significance level $\alpha = 0.05$ was used. All data analyses were done using Stata/SE 12 (StataCorp LP, College Station, TX, USA).

SUPPLEMENTARY FIGURES AND TABLES

		Conditioning intensity		
Parameter	Description	RIC (n=69)	RIC-UCB (n=18)	MAB (n=24)
Age, median(range)		55 (26-66)	53 (34-64)	32 (19-52)
Sex female (%)		31 (45)	6 (33)	10 (42)
Diagnosis				
	ALL	4	0	8
	AML	28	9	13
	CLL	6	1	0
	CML	2	2	2
	MDS	6	2	1
	MM	3	0	0
	NHL	9	1	0
	Other	11	3	0
Graft source				
	Sib	27	0	10
	MUD	42	0	14
	dUCBT	0	18	0
Conditioning regimen				
	Cyclo+TBI 12 Gy	0	0	21
	Cyclo+Busu	0	0	2
	Flu+TBI 12 Gy	0	0	1
	Flu+Cyclo+TBI 2x2 Gy	9	18	0
	Flu+TBI 2 Gy	57	0	0
	TBI 2 Gy	3	0	0

Supplementary Table 1. Patient and graft characteristics (n=111).

Sib= sibling donor; MUD= matched unrelated donor; dUCBT= double umbilical cord blood transplantation; Cyclo= cyclophosphamide; Busu= busulfan; Flu= fludarabine; TBI= total body irradiation



Supplementary Figure 1. Correlation plot between CEC numbers and CD34 numbers. All values were log transformed in order to compress the figure. Spearman correlation coefficient was calculated with non-normalized values.