Mesenchymal stromal cells from pooled mononuclear cells of multiple bone marrow donors as rescue therapy in pediatric severe steroid-refractory graft-*versus*-host disease: a multicenter survey

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Supplementary material

Methods

I. Recruitment of bone marrow donors and testing

Written informed consent was obtained for the collection of up to 250 ml of additional bone marrow for the purpose of MSC banking with the approval of the local Ethics Committee Nr. 275/09 and in full agreement with the Declaration of Helsinki. Only healthy volunteer donors from whom small-volume requests (pediatric recipients) had been made were considered for additional bone marrow collection.

Bone marrow was collected under general anesthesia via bilateral aspiration from the iliac crest. The bone marrow was anti-coagulated with 7–12% acid-citrate-dextrose (ACD-A) and 7–12 IU heparin per milliliter of aspirate. The infectious disease marker panel exceeded the minimal requirements of Joint Accreditation Committee–ISCT & EBMT and the German Stem Cell Act.

Bone marrow processing

BM-MNCs were enriched from the bone marrow aspirate by Ficoll (GE Healthcare, Munich, Germany) density centrifugation using the Sepax II NeatCell process (Biosafe, Eysins, Switzerland) according to the manufacturer's instructions. The cells from each donor were resuspended in cryomedium consisting of 5% human serum albumin (HSA) and 10% dimethyl sulfoxide (DMSO) in XVivo and were frozen individually in one bag and three back-up cryovials using a rate-controlled freezer (Schöllkrippen, Germany). The cells were then stored in the vapor phase of a liquid nitrogen tank (Tec-Lab, Idstein, Germany) until use.

II. Collection of platelet concentrates and generation of platelet lysates (PLs)

As a starting material for PLs, we used pooled platelets from 1-2-day-old buffy coats containing approximately 10% plasma in PASIII. The platelets were approved for clinical use in accordance with the German guidelines for blood products. Four to six platelet concentrates were pooled as one batch of PL, aliquoted in a class A in B environment into sterile 50 ml Falcon tubes and immediately frozen at -80 °C. Individual aliquots were thawed after at least 24 hours and centrifuged for 10 minutes at 3500 × *g*. The supernatants (PLs) were collected and subjected to extended release testing, including confirmation of freedom from bacteria (BacT/Alert, Biomerieux) and the potential of the PLs to promote MSC progenitor adherence and MSC expansion.

Processing facility

All processes were performed in accordance with full GMP criteria in the clean room suite (class A in B environment) at the Department of Cellular Therapeutics/Cell Processing of the German Red Cross Blood Service, which is in full compliance with a quality management system with formal permission from the state government [manufacturing license according to §20b/c (BM collection and testing) and §13 (MSC generation and testing) of the German Medicines Act].

III. Pre-testing before the establishment of the MSC bank

1. Testing of PLs

a) Potential of PLs to promote the adherence of MSC progenitors

Before using the PLs, we assessed the optimal concentration of PLs to promote the generation of MSCs from BM-MNCs and how the process of filtration through a 0.2 µm filter may affect this potential. Filtered and unfiltered PLs at a concentration of 5% or 10% (v/v) were used to supplement DMEM containing 5 IU/ml heparin. BM-MNCs were cultured in these media for 72 hours (4.3×10^6 BM-MNCs in 25 cm^2 flasks). After removal of the nonadherent cell fraction, the adherent cells were further cultured until the primary MSCs reached 80–90% confluence. The medium was replaced every 3–4 days. Primary MSCs were detached within 11–13 days using TrypLE (Life Technologies, Darmstadt, Germany). Cell viability was determined by Trypan blue staining.

b) Capacity of PLs to expand MSCs

PLs were also assessed for their capacity to expand primary MSCs. For this purpose, MSCs were plated in four 25 cm^2 culture flasks (5×10^4 MSCs/flask) in the following media: DMEM containing 5 IU/ml heparin supplemented with 10% unfiltered PL, DMEM supplemented with 10% filtered PL, DMEM supplemented with 5% unfiltered PL, or DMEM supplemented with 5% filtered PL. MSCs were detached at 80–90% confluence, and their expansion rate was estimated. The functional specification for the approval of PLs for use was that they had to support the doubling of the plated MSCs within 1 week in culture.

2. Generation of MSCs from back-up BM-MNC samples from each donor for additional tests

BM-MNCs from each donor were cultured ($1.71\times10^5/cm^2$) in 5% PL/DMEM for 72 hours. The nonadherent cell fraction was removed, and the adherent cells were cultured further in fresh medium for an additional 10 days until they reached 80–90% confluence. On day 13, the MSCs were harvested, and the cell number was determined using the Trypan blue exclusion method. The generated MSCs were frozen individually to compare the proliferative and

allosuppressive potentials of MSCs from each BM donor with those of clinical MEPs generated from the MSC bank.

IV. Generation of the MSC bank and clinical-grade MSC end-products (MEPs)

1. Generation of the MSC bank

BM-MNCs from each donor frozen in bags were thawed in a Plasmatherm device at 37 °C. The cells were washed, resuspended in 5% PL/DMEM, and pooled. A suspension containing 2.8×10^9 cells was plated in eleven 2-CellSTACKs (Corning, Amsterdam, The Netherlands) and one single CellSTACK. After 72 hours, the nonadherent cells were removed, and the adherent cells were cultured further for 11 days in 5% PL/DMEM until the MSCs were 80–90% confluent. During this period, the medium was replaced every 3–4 days. On day 14, before detachment of the primary MSCs, 5 ml of culture medium was collected from each CellSTACK and was pooled for sterility testing (for aerobic and anaerobic bacteria and fungi). Then, the cells were detached using TrypLE, washed, and centrifuged for 7 minutes at 400 × *g*. The cell pellets were resuspended in medium, and the cells were counted using the Trypan blue exclusion method.

A small number of primary MSCs were used for phenotype determination via flow cytometry. The remaining MSCs were centrifuged for 7 minutes at 400 × *g* and then resuspended in 5% HSA/DMEM to a density of 3×10^6 cells/ml. One volume of the cell suspension was mixed with one volume of cold cryomedium consisting of 20% DMSO and 5% HSA in DMEM. Therefore, the final concentration of MSCs was 1.5×10^6/ml, and the final concentrations of the cryopreservation agents were 10% DMSO and 5% HSA.

The MSCs were aliquoted into cryovials (each containing 1.5×10^6 MSC-P1) and then cryopreserved using a Cryoserve controlled-rate freezer according to established protocols. The frozen vials were stored in the vapor phase of a liquid nitrogen tank and were referred to as the MSC bank. The remaining MSCs were mixed with cryomedium and tested for sterility.

2. Generation of clinical-scale MEPs

To generate and validate clinical-scale MEPs, three randomly selected MSC-P1 aliquots were thawed 6–8 weeks after cryopreservation. After washing with culture medium containing 10% PL, the percentage of viable cells was assessed by trypan blue staining. The cells from one MSC bank vial were plated in one 1-CellSTACK (636 cm^2) and cultured in DMEM supplemented with 5 IU/ml heparin and 10% PL (v/v). The medium was replaced on day 4, and then, the MSCs were detached using TrypLE on day 6 or 7 and were plated at a density 2×10^3 cells/cm^2 in eight 2-CellSTACKs (1,272 cm^2 each) as passage 2. On day 6 or 7, the MSCs were detached as described for passage 1. The harvested MSCs were washed twice with a solution containing 0.5% HSA and 0.9% NaCl, and the number of MSCs was

counted by trypan blue staining. Further, MSCs were resuspended in cryomedium (0.9% NaCl containing 5% HSA and 10% DMSO) as passage 2 MEPs and were distributed in cryobags (each containing 1–3×10^6 MSCs/ml in 45 ml of cryomedium). The samples were cryopreserved using a Cryoserve controlled-rate freezer and were stored in the vapor phase of liquid nitrogen.

3. Validation of the MEPs

For this purpose, we thawed three MEPs (bags), washed the cells with 5% PL/DMEM, and then performed extensive quality testing without further expansion, i.e., on cells treated according to the procedure for MSC administration to patients*.*

a) Cell enumeration and viability of thawed MSCs

The total cell number (recovery) was quantified using a Neubauer hemocytometer. MSC viability was assessed using the Trypan blue method.

b) Phenotypic characterization

Flow cytometric analysis was performed using criteria exceeding the ISCT minimal criteria for MSCs[27]. To determine the phenotypes of thawed MSCs, the cells were stained with fluorochrome-conjugated mouse anti-human monoclonal antibodies (Supplementary Table 2). Fluorochrome-conjugated mouse immunoglobulins were used as isotype controls. Labeled MSCs were incubated for 30 minutes at 4 °C and then washed twice with PBS. To evaluate the viability of thawed MSCs, the cells were counterstained with propidium iodide prior to phenotypic characterization via flow cytometry. Immunostained MSCs were analyzed using a FACSCalibur flow cytometer (Becton-Dickinson) and using CellQuest software.

c) *Microbiological examinations (sterility, mycoplasma, and endotoxin levels in supernatants)*

The bioburden was assessed using the EP-conforming BacT/Alert method after formal matrix validation. Cultures were incubated for 7 days. Mycoplasma testing was performed via NAT, and the endotoxin levels were quantified via a modified limulus amoebocyte lysate assay using EP-conforming methods at a commercial laboratory. One MEP was tested by the accredited polymerase chain reaction (PCR) laboratory of Bioreliance (Glasgow, UK) for parvovirus B19, HIV I, HTLV-I and HTLV-II, hCMV, HBV, HCV, and human EV.

d) Evaluation of the allosuppressive potential of MEPs

To test the immunosuppressive effects of MEPs on an alloantigen-driven reaction, we used a standard two-way MLR as described previously[7].

e) Determination of senescence in three MEPs **in vitro**

To demonstrate that MEPs were mortal cells, we assessed their growth kinetics through 13 passages. To precisely estimate their growth kinetics, we calculated the number of population doublings (PDs) using the following formula:

PD for each subculture=[$\log_{10}$(NH) – $\log_{10}$(NI)] /$\log_{10}$(2), where NH is the number of harvested cells and NI is the initial number of cells.

f) Differentiation potential of MEPs

To evaluate their differentiation potential for adipogenesis, osteogenesis and chondrogenesis, three MEPs were thawed and cultured in appropriate tissue-specific induction media according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany) as previously reported[7].

g) Genetic analysis of clinical-grade MEPs

g.1) RT-PCR analysis of the expression of senescence genes in clinical-scale MEPs Quantification of the senescence genes p16 (CDKN2A) and p21 (CDKN1A) as well as p53, c-myc and hTERT via RT-PCR was performed as described previously[28;29]. Evaluation of the results was performed according to the study by Pfaffl[30].

g.2) Interphase fluorescence in situ hybridization (FISH)

Interphase FISH analysis was performed according to the manufacturer's protocol using the following probes for chromosomes 5 and 8: a two-color probe set for chromosome 5p15 (hTERT) and 5q35 (NSD1, Kreatech, Amsterdam, NL), and a three-color break-apart probe for chromosome 8q24 (MYC, Kreatech, Amsterdam, NL). Evaluation of the hybridization signals was performed using an automatic spot counting system (Applied Spectral Imaging, Edingen/Neckarhausen, Germany). For each probe, >300 nuclei were scanned and classified using a threshold of 5%.

g.3) Karyotypic analysis of MEPs

Chromosomal analysis of MEPs was performed according to standard cytogenetic methods using the GTG-banding technique. The chromosomes were analyzed, and the karyotype was described according to the International System for Cytogenetic Nomenclature (2013).

g.4) STR-PCR analysis of MEPs

The relative donor ratios in the MEPs were estimated via STR-PCR using a PowerPlex 16 Kit (Promega, Mannheim, Germany) by half-reaction amplification[31] and subsequent analyses by capillary electrophoreses using an Avant3100 device (Life Technologies, Darmstadt, Germany). Initially, each donor was genotyped to characterize informative STR alleles that are suitable to identify individual cells within a mixed sample. To estimate the relative donor ratios, the area under the peak curve was calculated as described previously[32].

V. Additional analysis after generation and validation of MEPs in the MSC bank

a) *Comparison of the proliferative and immunosuppressive effects of individual MSCs from the eight donors, pooled MSCs, and MSC bank aliquots*

To analyze the pooling effect of BM-MNCs, we thawed primary MSCs generated from each donor separately and cultured them for 7 days. After the detachment of MSC-P1, 4.4×10^4 MSCs were seeded in 25 cm^2 flasks as passage 2. Simultaneously, a proportion of MSCs from each donor was pooled (referred to as pooled-MSCs) and 4.4×10^4 of these pooled cells were cultured for 1 week. To compare the proliferative capacity of MSCs from the MSC bank with that of MSCs from each donor and of pooled MSCs, we cultured 4.4×10^4 passage 2 MSCs from four MSC bank aliquots. In parallel, we cultured the remaining cells to test their allosuppressive effect in an MLR. After detachment on day 7, the MSCs from each donor, pooled MSCs, and one MEP (MSC-140) were tested in an MLR.

To evaluate the immunosuppressive potential of frozen MSCs, we thawed back-up vials of six MEPs for assessment in an MLR.

VI. Documentation

Prior to generation of the MSC bank, processes for the testing and release of clinical specimens was formally validated in small-scale cultures based on standard operating protocols, batch records, testing instructions and protocols and according to formally defined specifications. A manufacturing license for an MSC bank and clinical specimens for use in clinical trials was obtained from the state government. Quality specifications were set after formal advisories were obtained from the Federal Drug Agency for biological medicines, the Paul Ehrlich Institute. The final cell products have been subject to all quality controls required for clinical use. Release criteria included: lack of detectable microbial contamination (aerobic or anaerobic bacteria, fungi, and mycoplasma) according to European pharmacopoeia, cell viability >70%, endotoxin levels in the final product ≤2.5 EU/ml, cell characterization with high expression of CD73, CD90, and CD105 (≥80%) and lack of expression of hematopoietic markers CD14, CD34 and CD45 (≤10%).

VII. Patients with steroid-refractory aGvHD.

Eighty-one MEPs generated from aliquots of the MSC bank were administered on a compassionate-use basis after approval of the regulatory authorities to 26 patients with refractory acute GvHD at a target dose of $1–2\times10^6$ MSCs/kg BW in 7 transplantation centers. The patients received HSC-PB or BM transplants for malignant (n=21; ALL, n=8; AML, n=5; MDS, n=6; and RMS, n=2) or non-malignant diseases (n=5; SCN, n=2; DBA, n=1; CGD, n=1; and SAA, n=1). Details of the patient characteristics are provided in Table 1.

The median time of transplantation after the onset of aGvHD was 22.5 days (range: 9-270 days). All patients suffered from severe steroid-refractory aGvHD (grade II, n=1; grade III, n=11; and grade IV, n=14) upon the initial MSC infusion. Patients were refractory to treatment with two (n=1), 3 (n=11), or four or more lines of GvHD treatment (n=14) before MSCs were administered. Therefore, our patient cohort comprised a very challenging population suffering from severe aGvHD, and in this cohort, the most frequently involved organs were the GIT (92.3%), the skin (61.5%), and the liver (34.6%; 66% of the patients with liver GvHD exhibited grade IV disease) (Table 2). Details of GvHD presentation, organ manifestation, the onset of GvHD, the interval from the onset of GvHD to treatment with MSCs, the MSC dose, the number of MSC transfusions and a detailed list of each patient's initial GvHD treatments are presented in Table 3.

VIII. Administration of the MSC bank end-products to patients

Passage 2 MSCs were thawed and immediately intravenously transfused to the patients within 15 min. The patients were not pre-medicated, and vital signs and oxygen saturation were monitored using a pulse oximeter for 2 hours after infusion.

As the application of MSCs was not performed in a prospective study but rather on the basis of emergency individualized patient treatment after the appropriate approvals, no strict infusion regimen was planned. Patients may have received several infusions according to their clinical response.

IX. Assessment of aGvHD

The severity of aGvHD (overall aGvHD grading) prior to the initiation of MSC therapy was assessed according to the Glucksberg-Seattle criteria as modified in the consensus conference in 1995.[33]

Using the same criteria, responses were assessed on day 28 after the initial MSC infusion and at the last follow-up.

Responses were defined as follows: CR, resolution of aGvHD in all involved organs; partial response (PR), a decrease of at least 1 GvHD stage in any 1 organ system without worsening in any other organ system; or no response (NR), no difference in any organ system or worsening in 1 or more organ systems. MSCs were transfused as a rescue therapy into all patients together with the preexisting GvHD treatment. Clinical status and response were assessed in all surviving patients as of May 15, 2015.

X. Statistics

The estimated median follow-up duration was obtained using a reverse Kaplan-Meier (KM) estimator. The data are expressed as the mean values ± SEM, and significance was assessed using Student's t-test. Categorical variables were compared between the response groups using Fisher's exact test or Kruskal-Wallis test.

OS probability was estimated using KM statistics. The OS time was calculated from the first MSC administration to death date or the last follow-up date. Non-relapse mortality (NRM) was defined as death of any cause without relapse or disease progression. Cumulative incidence (CI) of non-relapse mortality was performed considering relapse/disease progression as competing risk. The CI of NRM time was calculated from the first MSC administration to relapse/disease progression date, to death date and last follow-up for patients who relapsed, death without relapse and censored patients respectively.

All tests were two-tailed, and a p-value of less than 0.05 was considered to be statistically significant. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA) and the software for statistical computing and graphics R version 3.2.1 (R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/).

Supplementary tables and figures

Supplementary Table S1. Bone marrow donors used for the establishment of the MSC bank

Donor (sex)	**Age (years)**	**Volume of bone marrow (mL)**	**Relative proportion of each donor in the pool of all BM-MSCs**	**Frequency of $CD34^+$ cells among $CD45^+$ cells**
1 (male)	23.6	152.0	10.88%	0.97
2 (male)	21.6	176.8	11.98%	0.74
3 (male)	44.0	177.6	10.65%	2.30
4 (female)	30.1	181.6	12.11%	3.20
5 (male)	28.7	184.8	11.45%	3,92
6 (male)	23.8	184.8	17.29%	4.71
7 (male)	45.1	184.0	16.01%	2.26
8 (male)	44.0	182.4	9.58%	2.68

Supplementary Table S2. Antibodies used for the determination of the MSC phenotype

Antibodies	Company	Cat. Nr.	Clone	Isotype
IgG1 FITC	BioLegend	400109	MOPC-21	IgG1
IgG2a FITC	BioLegend	400209	MOPC-173	IgG2a
IgG1 PE	BioLegend	400113	MOPC-21	IgG1
IgG1 PerCP	BioLegend	400147	MOPC-21	IgG1
CD45 FITC	BioLegend	304005	HI30	IgG1
CD34 FITC	BioLegend	343603	561	IgG2a
CD14 FITC	BioLegend	325603	HCD14	IgG1
HLA-DR FITC	BioLegend	307603	L243	IgG2a
CD90 FITC	BioLegend	328107	5,00E+10	IgG1
CD73 PE	BioLegend	344003	V B-CD73.3	IgG1
CD105 PE	BioLegend	323205	43A3	IgG1
PI staining solution	BD Pharmingen	556463		

PI: propidium iodide

Supplementary Table S3. The individual patient data

Patient	Diagnosis	Age at SCT (years)	Sex	Conditioning regimen	Serotherapy	Graft	GvHD prophylaxis	Donor	HLA
1	ALL	9	m	Clof-Cyc-Eto-Flu-Thio-Mel	Campath	PBSC	no	FD	5/10
2	ALL	1	m	Clof-Cyc-Eto-Flu-Thio-Mel	Campath	PBSC	no	FD	6/10
3	ALL	3	m	TBI-Eto	no	BM	CSA	MSD	10/10
4	ALL	12	m	TBI-Eto	no	BM	CSA	MSD	10/10
5	ALL	8	m	TBI-Eto	ATG	PBSC	CSA+MTX	UD	9/10
6	ALL	5	m	TBI-Eto	ATG	BM	CSA+MTX	UD	10/10
7	ALL	17	f	Treo-Flu-Thio	ATG	BM	CSA	UD	10/10
8	ALL	2	f	Treo-Flu-Thio	ATG	CB	CSA+MMF	FD	4/6
9	AML	3	f	Bu-Cyc	ATG	PBSC	CSA+MTX	UD	10/10
10	AML	8	m	Bu-Cyc-Mel	no	BM	CSA	MSD	10/10
11	AML	10	m	Clof-Cyc-Eto-Flu-Thio-Mel	Campath	PBSC	no	FD	5/10
12	AML	7	m	Flu (recond. 3rd Tx)	Campath	PBSC	no	FD	6/10
13	AML	19	m	TBI-Flu-Mel	ATG	PBSC	CSA+MMF	UD	10/10
14	DBA	3	m	Flu-Thio-Mel	ATG	BM	CSA+MTX	UD	10/10
15	CGD	4	m	Bu-Flu	Campath	BM	CSA	UD	10/10
16	MDS	6	f	Bu-Cyc-Mel	no	BM	CSA	MSD	10/10
17	MDS	13	f	Bu-Cyc-Mel	ATG	BM	CSA+MTX	UD	8/10
18	MDS	1	f	Treo-Flu-Thio	no	BM	CSA+MMF	UD	10/10
19	MDS	8	m	Treo-Flu-Thio	ATG	PBSC	CSA+MTX	UD	9/10
20	MDS	4	f	Treo-Flu-Thio	ATG	PBSC	MMF	FD	5/10
21	MDS	6	m	Treo-Flu-Thio	ATG	BM	CSA+MTX	UD	10/10
22	RMS	13	f	Clof-Cyc-Eto-Flu-Thio-Mel	Campath	PBSC	no	FD	5/10
23	RMS	12	m	Clof-Cyc-Eto-Flu-Thio-Mel	Campath	PBSC	no	FD	5/10
24	SAA	11	m	Flu-Cyc	ATG	BM	CSA+MTX	UD	9/10
25	SCN	1	f	Treo-Flu-Thio	ATG	BM	CSA+MTX	UD	9/10
26	SCN	2	f	Treo-Flu-Thio	ATG	PBSC	CSA+MTX	UD	9/10

Abbr.: DBA, Diamond Blackfan anemia; CGD, chronic granulomatous disease; MDS, myelodysplastic syndrome; RMS, rhabdomyosarcoma; SAA, severe aplastic anemia; SCN, severe congenital neutropenia; SCT, stem cell transplantation; GvHD, graft-versus-host disease; Clof, clofarabine; Cy, cyclophosphamide; Eto, etoposide; Flu, fludarabine; Thio, thiotepa; Mel, melphalan; Bu, busulfan; Treo, treosulfan; Tx, transplantation; ATG, anti-thymocyte globulin; PBSC, peripheral blood stem cells; BM, bone marrow; CB, cord blood; CSA, cyclosporine A; MTX, methotrexate; MMF, mycophenolate mofetil; FD, family donor; MSD, matched sibling donor; UD, unrelated donor; HLA, human leukocyte antigen.

Supplementary Table S4. Influence of clinical factors on the treatment response

		Response at day 28				
	n (%)	CR (n=5)	PR (n=15)	NR (n=4)	Death (n=2)	*P*
	26 (100%)	19%	58%	15%	8%	
Sex						
female	10 (38%)	2	6	1	1	.852
male	16 (62%)	3	9	3	1	
Age						
median (range) years		7(2-19)	1 (8-17)	3 (1-6)		.244
0 - 2	5 (19%)	1	3	1		.686
> 2 - 12	17 (65%)	3	10	3	1	
>12 - 18	3 (12%)	0	2	0	1	
> 18	1 (4%)	1	0	0		
Diagnosis						.291
malignant disease	21 (81%)	4	13	2	2	
non-malignant disease	5 (19%)	1	2	2		
Donor						.344
MSD	4 (15%)	1	1	1	1	
FD	8 (31%)	1	7	0		
UD	15 (54%)	3	7	3	1	
Conditioning regimen						.782
TBI/Eto	5 (19%)	0	3	1	1	
Treo/Flu/TT	8 (31%)	1	5	2		
Bu/Cy/Mel	5 (19%)	2	2	0	1	
Clof/Cyc/Eto/Flu/Thio/Mel	8 (31%)	2	5	1		
Serotherapy						.778
without	5 (19%)	1	1	2	1	
ATG	14 (54%)	3	2	8	1	
Campath	7 (27%)	1	0	6		
Graft						.113
BM	13 (50%)	1	6	4	2	
PBSC	12 (46%)	4	8	0		
CB	1 (4%)	0	1	0		

		Response at day 28				
	n (%)	CR (n=5)	PR (n=15)	NR (n=4)	Death (n=2)	*P*
	26 (100%)	19%	58%	15%	8%	
GvHD prophylaxis						.892
without	6 (23%)	1	5	0		
CSA	6 (23%)	1	3	1	1	
CSA+MTX	10 (38%)	2	4	3	1	
CSA+MMF	3 (12%)	1	2	0		
MMF	1 (4%)	0	1	0		
aGvHD onset						.357
median (range) days		23 (10-270)	29 (12 - 140)	17 (9 -30)		
aGvHD overall grade						.321
II	1 (4%)	0	1	0		
III	8 (29%)	3	4	0	1	
IV	17 (65%)	2	10	4	1	
aGvHD treatment						.902
2 Lines	1 (4%)	0	1	0		
3 Lines	11 (42%)	2	7	1	1	
> 3 Lines	14 (54%)	3	7	3	1	
Number of infusions						.822
1 infusion	4 (15%)	2	2	0		
2 infusions	8 (31%)	1	3	2	2	
3 infusions	2 (8%)	0	2	0		
4 infusions	9 (35%)	1	6	2		
> 4 infusions	3 (12%)	1	2	0		
Mean MSCs/kg BW per infusion ($x10^6$)						.145
median (range)		1.8 (1.4 - 4)	2.1(0.9 - 7.7)	3.3 (2.6 - 4.4)		
Cumulative dose of MSCs /kg BW ($x10^6$)						.159
median (range)		5.3(1.4-9.2)	6 (2 - 31)	9.6 (6.6 -18)		

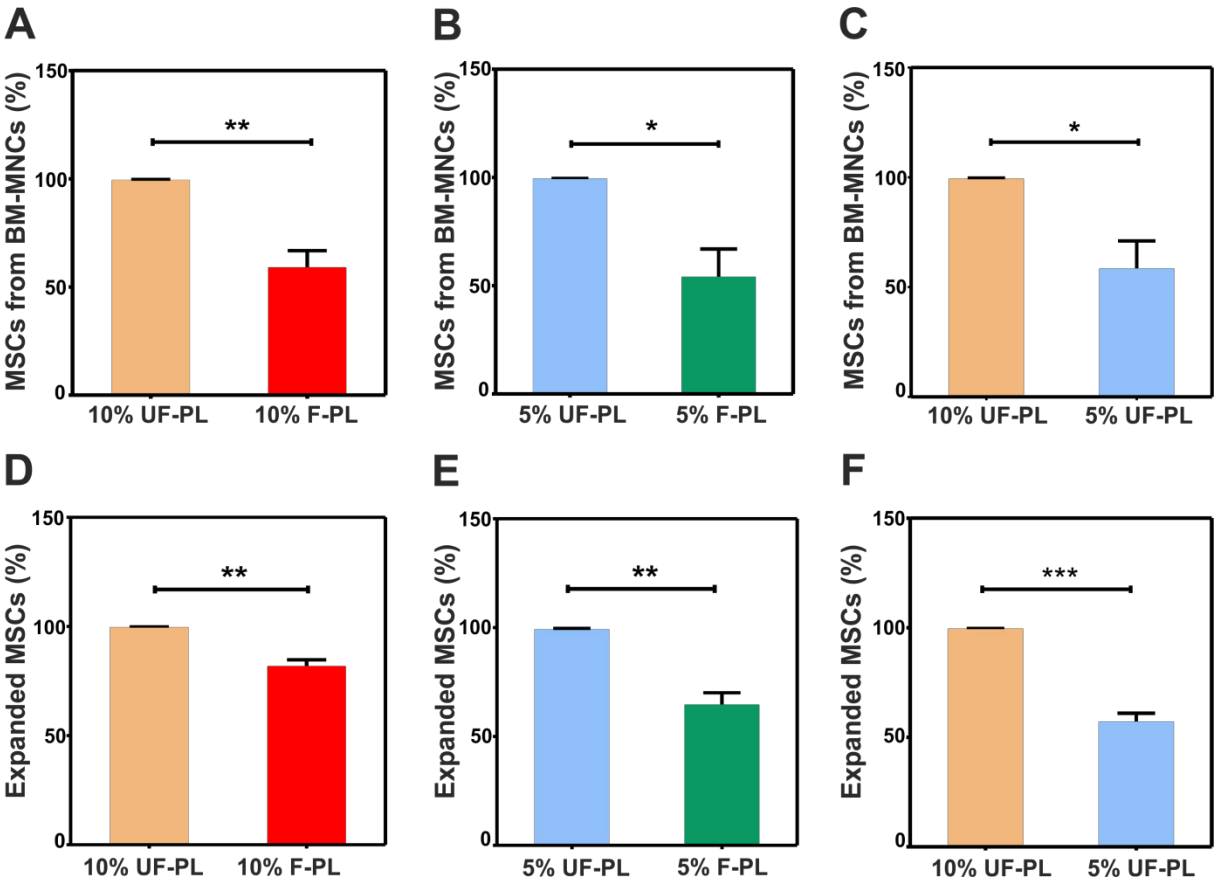
Abbr.: MSD, matched sibling donor; FD, family donor; UD, unrelated donor; TBI, total body irradiation; Eto, etoposide; Treo, treosulfan; Flu, fludarabine; Thio, thiotepa; Bu, busulfan; Cyc, cyclophosphamide; Mel, melphalan; Clof, clofarabine; ATG, antithymocyte globulin; BM, bone marrow; PBSC, peripheral blood stem cell; CB, cord blood; aGvHD, acute graft-versus-host disease; CSA, cyclosporine A; MTX, methotrexate; MMF, mycophenolate mofetil. * 2 patients died prior to day 28 and therefore they were excluded from analysis. Statistical analysis was performed using Fisher's Exact Test for categorical variables or the Kruskal-Wallis test for continuous variables.

Supplementary Table S5. Clinical response and survival outcome in pediatric patients with acute GvHD after treatment with MSC

Publication		GVHD	Response at day +28	OS observation time	OS Mean [95% CI]
Lucchini et al. [30]	n=11	aGVHD I-II: n=4 (36%) ; III-IV: n=4 (36%) ; cGVHD: n=3 (n=27%)	CR=23.8%; PR=47.6%;OR=71.4%	8 [4 -18] mo	8/11 = 73%
*only aGVHD	n=8	aGVHD I-II: n=4 (50%) ; III-IV: n=4 (50%)	CR=37.5%; PR=25%; OR=62.5%		5/8=62.5%
Prasad et al. [25]	n=12	aGVHD III: n=5 (42%); IV: n=7 (58%)	CR=17%; PR=50%; OR= 67%	2 years-OS	40% [20 - 82%]
Introna et al. [31]	n=15	aGVHD II: n=9 (60%), III-IV: n=3 (20%); chronic GVHD: n=3 (20%)	CR=46.7%; PR=20%; OR=66.7%	2 years-OS	52.5% [27-78%]
*only aGVHD	n=12	aGVHD I-II: n=9 (75%) ; III-IV: n=3 (25%)	CR=41.6%; PR=25%; OR=66.6%		
Le Blanc et al. [50]	n=25		CR=68%; PR=16%; OR=84%* at 6 weeks after infusions	2 years-OS	45 % [23-67%]
Kurtzberg et al. [26]	n=75	Grad B: n=9 (12%); C: n=21 (28%); D: n=45 (60%)	OR=61.3%	day +100 for OS	57.3%
Kuçi et al.	n=26	aGVHD II: n=1 (4%); III: n=11(42.3%); IV:n=14 (53.8%)	CR=19%; PR= 58%; OR=77%	2 years-OS	71.4% [53.6-95.3%]

Abbr.: aGvHD, acute graft-versus-host disease; CR, complete response; PR, partial response; OR, overall response; OS, overall survival; CI, confidence interval

Supplementary Figure S1. Evaluation of the effect of different concentrations and the filtration status of PLs on the generation and expansion of MSCs. (**A**) Effect of 10% filtered PL on MSC generation compared with 10% unfiltered PL (n=4). (**B**) Comparison between unfiltered and filtered PLs at a concentration of 5% with respect to MSC generation (n=4). (**C**) Influence of unfiltered PL at different concentrations on MSC generation (n=4). (**D**) Effect of 10% unfiltered PL on MSC expansion compared with 10% filtered PL (n=3). (**E**) Effect of 5% unfiltered PL on MSC expansion compared with 5% filtered PL (n=3). (**F**) Unfiltered PL at a concentration of 10% was significantly more efficient for the expansion of MSCs than unfiltered PL at a concentration of 5% (n=5). The results are presented as the mean values ± SEM. UF=unfiltered; F=filtered. Statistical analysis was performed using Student's t-test.

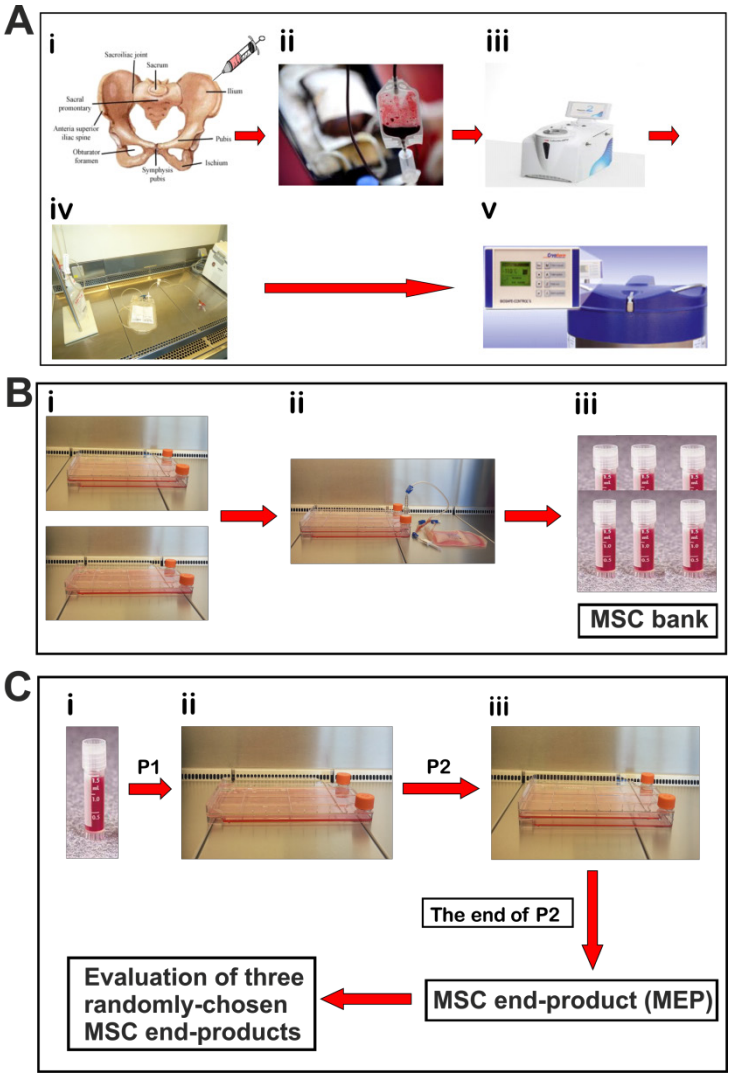


Supplementary Figure S2. Establishment of the MSC bank.

(**A**) Collection of bone marrow and separation of BM-MNCs. (**Ai**) Bone marrow was collected from the iliac crest and transferred to bags (**Aii**). (**Aiii**) BM-MNCs were isolated by Ficoll (Sepax) gradient centrifugation, frozen individually in bags (**Aiv**), and stored in liquid nitrogen (**Av**).

(**B**) Generation of the MSC bank from pooled BM-MNCs. (**Bi**) Pooled BM-MNCs from eight donors were plated in 11 2-CellSTACKs (2.5×10^8 cells each) and a single CellSTACK (1.25×10^8 cells). After 72 hours, the nonadherent cells were removed. (**Bii**) On day 14 in culture, primary MSCs were harvested and frozen in 209 cryovials (containing 1.5×10^6 MSCs each). These aliquots constituted the MSC bank (**Biii**).

(**C**) Generation of MSC end-products (MEPs) (**C**i) Three randomly selected cryopreserved vials of MSCs from the MSC bank were thawed. (**Cii**) Cells were plated in a single CellSTACK and cultured for 6–7 days. (**Ciii**) On day 6 or 7 (end of passage 1), the MSCs were harvested and plated in eight 2-CellSTACKs as passage 2 and cultured for an additional week. The expanded MSCs were harvested and cryopreserved as MEPs.



Supplementary Figure S3. Phenotypic and functional testing of MEPs

(**A**) Phenotype of MEPs at the end of passage 2, as determined by flow cytometry. (**B**) Allosuppressive potential of three MEPs in an MLR. Representative patterns of MEPs differentiation into adipocytes (**C**), osteoblasts (**D**) or chondrocytes (**E**) after their culture in the tissue-specific induction media.