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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ABSTRACT

To circumvent donor-to-donor heterogeneity which may lead to inconsistent results after treatment of acute graft-*versus*-host disease with mesenchymal stromal cells generated from single donors we developed a novel approach by generating these cells from pooled bone marrow mononuclear cells of 8 healthy "3rd-party" donors. Generated cells were frozen in 209 vials and designated as mesenchymal stromal cell bank. These vials served as a source for generation of clinical grade mesenchymal stromal cell end-products, which exhibited typical mesenchymal stromal cell phenotype, trilineage differentiation potential and at later passages expressed replicative senescence-related markers (p21 and p16). Genetic analysis demonstrated their genomic stability (normal karyotype and a diploid pattern). Importantly, clinical end-products exerted a significantly higher allosuppressive potential than the mean allosuppressive potential of mesenchymal stromal cells generated from the same donors individually. Administration of 81 mesenchymal stromal cell end-products to 26 patients with severe steroid-resistant acute graft-*versus*-host disease in 7 stem cell transplant centers who were refractory to many lines of treatment, induced a 77% overall response at the primary end point (day 28). Remarkably, although the cohort of patients was highly challenging (96% grade III/IV and only 4% grade II graft-*versus*-host disease), after treatment with mesenchymal stromal cell end-products the overall survival rate at two years follow up was 71±11% for the entire patient cohort, compared to 51.4±9.0% in graft-*versus*-host disease clinical studies, in which mesenchymal stromal cells were derived from single donors. Mesenchymal stromal cell end-products may, therefore, provide a novel therapeutic tool for the effective treatment of severe acute graft-*versus*-host disease.

Introduction

Since the first clinical trial of mesenchymal stromal cells (MSC) in 1995,[1] their use has expanded rapidly. To date, 561 registered clinical trials (retrieved from *www.clinicaltrials.gov*, 2nd December 2015) have been performed to examine an extremely wide spectrum of therapeutic MSC applications. Despite the general consensus that MSCs appear to be well-tolerated, safe and effective for the treatment of various diseases, there has been limited progress in this field due to inconsistencies in the outcome of clinical trials. These inconsistencies may be attributed to the lack of a standardized methodology for MSC generation[2] and MSC dosing, the heterogeneity in MSC potency between donors[3] and tissue sources,[4] and the variable number of MSC progenitor cells between tissue samples.[5] MSCs exhibit donor-specific variations in their immunosuppressive properties not only at the donor level,[3,6] but also at the clonal level.[7] A recent study demonstrated passage effect on the immunosuppressive effect of MSCs by obtaining an optimal immunosuppressive effect in patients with steroid-resistant acute graft-*versus*-host disease (aGvHD) after administration of MSCs at passage 1 or 2.[8] In contrast, several other reports demonstrated that the immunosuppressive effect of MSCs remains unchanged for up to 7 or 8 passages in culture.[9,10] Another important issue regarding the clinical application of MSCs is their culture under serum-free conditions. The majority of clinical studies have used MSCs that were expanded in media supplemented with fetal bovine serum (FBS).[1,11-15] To avoid the risks associated with the use of FBS,[16] platelet lysate (PL) was proposed as a supplement to tissue culture media for MSCs.[17] Recently, several studies showed that MSCs that were expanded in PL exhibited the same efficacy as MSCs cultured in serum-containing media for the treatment of GvHD.[18-22]

To date, clinical studies have used MSCs that have been generated from several individual donors. Considering the aforementioned inter-donor heterogeneity and the need for a large number of "off-the-shelf" MSCs, the establishment of MSC banks appears to be an indispensable strategy for providing a continuous supply of MSCs with predictable potency. To our knowledge, there are few established MSC banks worldwide, and these MSC banks were generated by separately isolating, expanding, and freezing MSCs from up to 10 donors in FBS-containing media.[23-26]

In the current study, we report for the first time the establishment of a serum-free and GMP-compliant MSC bank generated from pooled bone marrow mononuclear cells (BM-MNCs) of multiple donors as a novel strategy to circumvent donor-to-donor variability. Clinical-grade MSC endproducts (MEPs) derived from the MSC bank were thoroughly assessed for their proliferation, differentiation, and, in particular, for the allosuppressive potential *in vitro*. Importantly, 81 MEPs were administered as a rescue therapy to 26 pediatric patients with severe steroid-refractory aGvHD in seven transplantation centers. Safety and efficacy of MEPs was compared to MSCs generated from a single or several individual donors that have been used in the GvHD-clinical studies reported thus far.

Methods

Generation of MSC bank and clinical-grade MEPs

Bone marrow was collected from 8 healthy volunteers (age 21-45 years old) after written informed consent and after the approval of the local Ethics Committee (n. 275/09). BM-MNCs were enriched from the bone marrow aspirate by using the Sepax II NeatCell process (Biosafe, Eysins, Switzerland) and frozen individually. After thawing and washing these BM-MNCs were pooled. This pool of BM-MNCs from 8 donors was used to generate MSCs over 14 days in culture. After their detachment, passage 1 mesenchymal stromal cells (MSC-P1) were washed and aliquoted into 209 cryovials (each containing 1.5×10^6 MSC-P1). Cryopreserved vials with MSC-P1 were referred to as the MSC bank.

To generate clinical-grade MEPs, MSC-P1 aliquots from the MSC bank were thawed and after washing they were expanded in medium containing 10% PL till the end of passage 2. These MSCs were re-suspended in cryomedium (0.9% NaCl containing 5% HSA and 10% DMSO), distributed in cryobags (each containing $1-3 \times 10^6$ MSCs/mL in 45 mL of cryomedium) and frozen in liquid nitrogen until use.

Further details on methods as to generation of MSC bank from BM-MNC pool, including collection and testing of platelet lysates, validation of MEPs regarding their phenotype (flow cytometry), differentiation and allosuppressive potential, genetic analysis (cytogenetics, FISH, RT-PCR, STR-PCR), determination of senescence and its typical markers, characteristics of patients with severe aGvHD, assessment of the disease and its treatment with MEPs and statistics, are presented in the *Online Supplementary Appendix*.

Results

Collection of bone marrow from 8 healthy 3rd-party donors and isolation of BM-MNCs

After obtaining written informed consent, healthy donors donated 152-184 mL of bone marrow (*Online Supplementary Table S1*). Following isolation of the BM-MNCs using the Sepax method, a total of 10.82×10^9 BM-MNCs were collected from 8 donors. The absolute number of BM-MNCs per 1 mL of bone marrow after isolation was $4.1 \times 10^6 \pm 7.8 \times 10^5$. All donors were equally represented in the BM-MNC pool, i.e. the relative contribution of BM-MNCs by each donor was 12.5±2.7% (*Online Supplementary Table S1*). The cells from each donor were re-suspended in cryomedium and frozen individually in bags. The total number of frozen BM-MNCs was 9.86×10^9.

Testing of the concentration of PL for the generation and expansion of MSC

Before the establishment of the MSC bank, we determined the optimal concentration of PL to support the adherence of MSC progenitors and to assess whether PL filtration was needed. We observed that unfiltered PL at a concentration of 10% (*Online Supplementary Figure S1A*) or 5% (*Online Supplementary Figure S1B*) was optimal for MSC generation compared with media supplemented with filtered PL at the same concentrations ($P<0.002$ and $P<0.01$, respectively). In addition, 10% PL was significantly more efficient for MSC generation by plastic adherence ($P<0.02$) (*Online Supplementary Figure S1C*). Evaluation of the capacity of PL to expand MSC revealed that unfiltered PL at a concentration of 10% (*Online Supplementary Figure S1D*) or 5% (*Online Supplementary Figure S1E*) induced significantly greater expansion of MSC than filtered PL at the corresponding concentration ($P<0.0008$ and $P<0.003$, respectively). Furthermore, unfil-

tered PL at 10% was significantly more effective for expansion of MSC than 5% unfiltered PL (*P*<0.0001) (*Online Supplementary Figure S1F*).

Establishment of the MSC bank and generation of clinical-scale MEP

After thawing, the number of pooled BM-MNCs was 2.8×10^9. Culture of these cells for 14 days resulted in 320×10^6 primary MSC which exhibited a viability of 98.9% and expressed consensus markers for MSC. These cells were designated as MSC-P1 (passage 1) and were aliquoted and frozen in 209 cryovials, representing the MSC bank (*Online Supplementary Figure S2Bi-iii*).

To generate clinical-scale MEP and validate their proliferative, differentiation and allosuppressive potential, three randomly selected MSC aliquots from the MSC bank were thawed after cryopreservation for 6-8 weeks. The mean cell recovery was 1.39×10^6 (range $1.23-1.48 \times 10^6$) viable cells/vial, and the viability of these cells was 95.25±1.73% (range 93.45%-96.9%). On average, the expansion of these MSC over two weeks until the end of passage 2 resulted in the generation of 4.7×10^8 viable MSC

(range $4.2-5.48 \times 10^8$), in which the number of cumulative population doublings (CPD) was 8.5±0.04. These samples (units), referred to as clinical-scale MEP, were frozen in 4-7 bags containing $50-129 \times 10^6$ MSC until use. These units served as clinical doses for recipients of various body weights (*Online Supplementary Figure S2Ci-iii*). Before freezing, the MEP phenotypes were analyzed to assess whether the samples fulfilled the International Society for Cellular Therapy (ISCT) MSC criteria. They were sterile, were mycoplasma-negative and contained endotoxin levels below the limit of detection (<0.2 EU/mL). For quality control and release purposes, all bags generated from one aliquot were considered as one batch.

Validation of the MEP: phenotype, allosuppressive and differentiation potential

For quality assessment, the MEP from three different batches were thawed after 4-6 weeks of cryopreservation. After thawing, 87.9±3.6% of the cells were viable, representing a mean recovery of 78.5±9.8%. MEP were negative for HLA-DR and the hematopoietic markers CD45, CD14, and CD34. However, they expressed high levels of
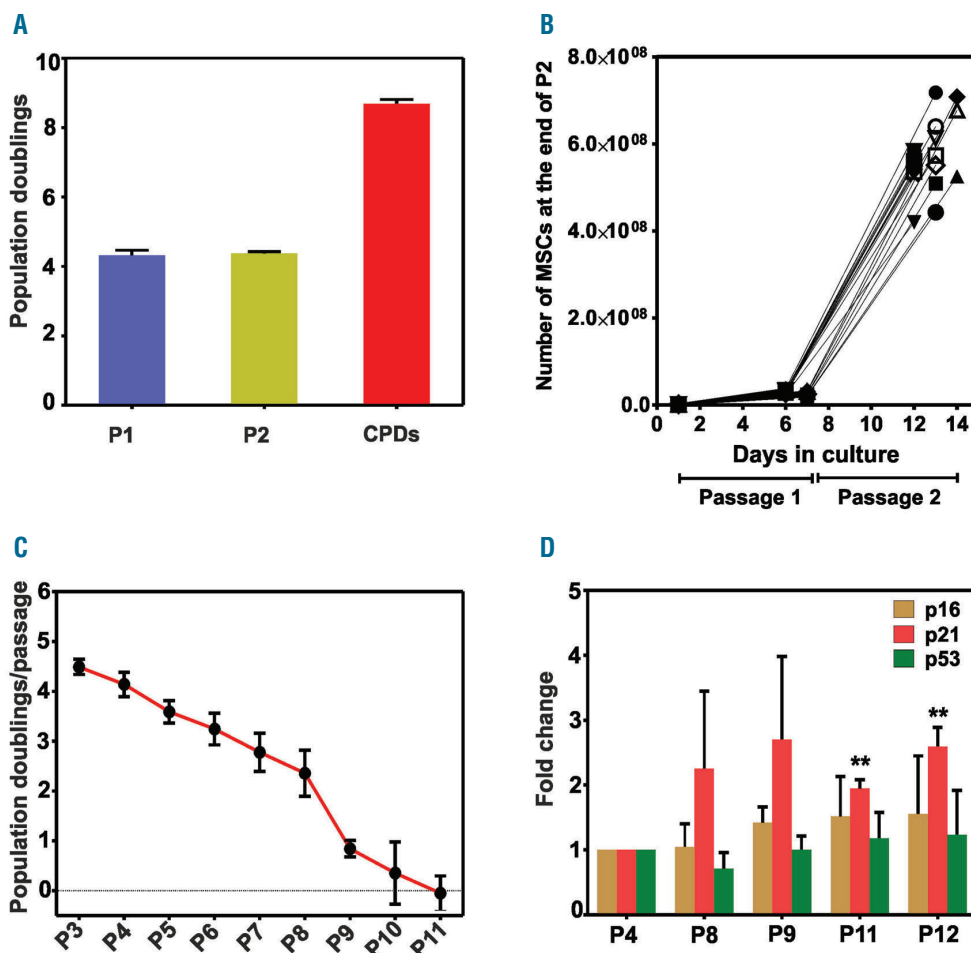

Figure 1. Proliferation potential and senescence of mesenchymal stromal cells (MSC) and MSC end-products (MEP). (A) MSC proliferated at a rate of approximately four population doublings (PD) per passage. The number of cumulative PD (CPD) was 8.5±0.4. Data presented as mean±SEM (n=3). (B) *Ex vivo* expansion of nineteen MEP through 2 passages. (C) Expansion of MEP for 11 passages and estimation of the number of PD (n=3). (D) RT-PCR analysis of genes related to cell senescence in three clinical-scale MEP. Data presented as mean±SEM (n=3). **P<0.003. Statistical analysis was performed using Student's *t*-test.

the consensus MSC markers CD73, CD90 and CD105 and HLA class I molecules (*Online Supplementary Figure 3B*). The mean inhibitory effect of thawed MEP on the proliferation of HLA-mismatched peripheral blood MNC (PB-MNC) was 36.7±3.2% (*Online Supplementary Figure S3B*). All thawed MEP differentiated into adipocytes, osteoblasts or chondrocytes (*Online Supplementary Figure S3C-E*).

Proliferation potential and senescence of MEP

Three MSC bank cryovials were expanded to end-products, and these samples demonstrated a similar number of population doublings (PD) in passages 1 and 2 (4.3 PD/passage). The number of cumulative PD (CPD) did not exceed 8.5±0.024 (Figure 1A). In the interim, we have expanded 19 MSC-aliquots up to the end-product. The mean viability of these thawed aliquots was 95.1±2.1% (range 88.8%-98.2%). As shown in Figure 1B, the mean cell number of all expanded MEP at the end of passage 2 was $5.64 \times 10^8 \pm 0.78 \times 10^8$ MSC, indicating a predictable proliferation potential.

To test whether the MSC were immortalized during expansion, we expanded three MEP for 13 passages. As shown in Figure 1C, at some point between passages 5 and 11, the MSC underwent replicative senescence, and the number of PD diminished rapidly. The three MEP that were expanded for 11 passages underwent 30.2 CPDs in 68 days. MEP expansion until passage 13 exhibited no post-senescence proliferation (*data not shown*).

Moreover, analysis of senescence marker expression in three clinical-scale MEP demonstrated no significant elevation in the levels of p16 and p53 gene expression. In contrast, p21 gene expression was significantly increased at passages 11 and 12 compared with passage 4 (Figure 1D). Consistent with the senescent behavior of the MSC from our bank, none of the three examined MEP expressed the oncogene c-myc or hTERT (*data not shown*).

Allosuppressive potential of MSC isolated from individual donors and MEP

Based on our preliminary data, we hypothesized that MSC generated from pooled BM-MNC of 8 donors may exhibit a higher allosuppressive potential than MSC generated from individual donors or pooled MSC from different donors. To test our hypothesis, we expanded MSC from the 8 individual donors from the start of passage 2, and pooled MSCs from the 8 donors (pooled-MSCs) or one MEP (MSC-140) until the end of passage 2. As expected, the allosuppressive potential of the MSC from individual donors in mixed leukocyte reaction (MLR) was highly heterogeneous, ranging from 20% (donors 1 and 8) to approximately 80% (donors 2 and 3) (Figure 2A). The allosuppressive potential of the pooled-MSC was equal to the mean allosuppressive potential of the MSC from the 8 individual donors. In contrast, the allosuppressive potential of the expanded MSC-140 end-product from the MSC bank was significantly greater than that of the pooled-MSC or the mean allosuppressive potential of the MSC from the 8 individual donors (*P*<0.001 and *P*<0.01, respectively). These results show the advantage of pooling BM-MNC for MSC generation. In addition, the allosuppressive potential of six freeze-thawed MEP (as usually administered to patients) demonstrated a consistent allosuppressive effect *in vitro* (Figure 2B), indicating the equipotency of MSC batches (mean 52±8.7%).

Table 1. Patients' characteristics.

	N	%
Patients enrolled	26	100
Sex		
Female	10	38
Male	16	62
Age		
Median [range] years	6.5 [1 - 19]	
Diagnosis		
ALL	8	31
AML	5	19
MDS	6	23
RMS	2	8
SCN/SAA/CGD/DBA	2/1/1/1	8/4/4/4
SCT source		
BM	13	50
PBSC	12	46
CB	1	4
Donor		
MSD	4	15
FD	8	31
UD	14	54
Conditioning regimen		
TBI-based	5	19
Chemotherapy-based	21	81
Serotherapy		
ATG	14	54
Campath	7	27
Without serotherapy	5	19
GvHD prophylaxis		
CSA	6	23
CSA+MTX	10	38
CSA+MMF	3	12
MMF	1	4
Without prophylaxis	6	23

MDS: myelodysplastic syndrome; RMS: rhabdomyosarcoma; SCN: severe congenital neutropenia; SAA: severe aplastic anemia; CGD: chronic granulomatous disease; DBA: Diamond-Blackfan anemia; SCT: stem cell transplantation; BM: bone marrow; PBSC: peripheral blood stem cells; CB: cord blood; MSD: matched sibling donor; FD: family donor; UD: unrelated donor; TBI: total body irradiation; CSA: cyclosporine A; MTX: methotrexate; MMF: mycophenolate mofetil.

Genetic characterization of MEP

Because *in vitro* culture may cause chromosomal aberrations in cells, we performed chromosomal analysis of 25 MSC undergoing mitosis with a resolution of approximately 350-400 bands; 21 of the 25 analyzed metaphases demonstrated a normal karyotype (Figure 3A). Four out of the 25 analyzed metaphases displayed balanced translocation between the short arms of chromosomes 5 and 19. Breakpoints were identified in bands 5p13 and 19p13.3. The karyotype was mos 46,XY[21]/46,XY,t(5;19)(p13;p13.3)[4]. FISH analysis using a 2-color probe for chromosome 5p15 (hTERT) and 5q35 (NSD1) and a 3-color break-apart probe for the MYC gene at chromosomal locus 8q24 demonstrated that the majority of MEP possessed a normal diploid pattern (Figure 3B and C). Interphase nuclei after 2-color hybridization of probe sets 5p15 (green) and 5q35 (red) revealed that 97.2% of the cells showed a normal diploid pattern for chromosome 5, and that only 2.8% of the cells showed a tetraploid hybridization pattern (Figure 3D). Similarly, visualization of interphase nuclei after 3-color hybridization of the MYC break-apart probe (Figure 3C) showed that 97% of the MSCs carried two normal fusion signals

Table 2. Acute graft-*versus*-host disease and mesenchymal stromal cell treatment.

Patient	aGvHD onset (days)	aGvHD duration prior to MSC (days)	aGVHD at initial MSC treatment aGVHD overall grade	involved organs and stages	N. of infusions	Mean cells/kg BW per infusion (x10⁶)	Cumulative dose of cells/kg BW (x10⁶)	Prior and concurrent immunosuppression
1	77	32	IV	GI IV/Skin IV/Liver IV	2	1.7	3.5	Steroids, MMF, CSA
2	70	13	III	GI III/Skin III	5	3.5	17.0	Steroids, MMF, CSA, ECP
3	12	34	III	GI IV	4	3.2	18.1	Steroids, MMF, CSA, Etanercept
4	22	8	IV	Skin IV	2	1.5	3.0	Steroids, MMF, CSA
5	29	29	IV	GI IV/Skin IV/Liver II	2	2.0	4.0	Steroids, Budesonide, Tacrolimus
6	13	22	III	GI III/Skin III/Liver III	4	2.6	10.4	Steroids, MMF, CSA
7	21	19	IV	GI IV/Skin IV/Eyes IV	4	0.9	3.5	Steroids, MMF, ECP
8	12	56	II	GI I-II	1	6.0	6.0	Steroids, MMF, CSA
9	29	21	III	GI III/Liver III	4	1.5	6.0	Steroids, MMF, CSA, Tacrolimus
10	23	23	III	GI III	5	1.8	9.2	Steroids, MMF, CSA, ECP
11	81	14	III	GI III/Skin III/Eyes III	4	2.1	8.4	Steroids, MMF, CSA
12	270	19	III	GI II/Skin II	2	2.6	5.3	Steroids, MMF
13	10	42	III	GI III/Skin III	1	1.4	1.4	Steroids, MMF, CSA, Etanercept, Pentostatin, ECP
14	22	9	III	GI IV	2	3.3	6.6	Steroids, MMF, CSA, Basiliximab, Sirolimus
15	32	112	IV	GI IV/Skin IV	2	2.5	5.0	Steroids, MMF, MTX, Etanercept, Infliximab
16	21	7	IV	GI IV/Skin II/Liver IV	3	2.5	7.6	Steroids, MMF, CSA, Everolimus, ECP
17	30	10	IV	GI IV/Skin IV	2	1.0	2.0	Steroids, MMF, CSA
18	13	96	IV	GI IV/Skin IV	4	7.7	31.0	Steroids, MMF, CSA, Infliximab, Basiliximab, ECP
19	15	9	IV	GI IV/Skin IV/Liver IV	4	1.6	6.4	Steroids, MMF, CSA
20	83	53	III	GI III	1	2.0	2.0	Steroids, Infliximab, Tacrolimus
21	30	54	III	GI IV	4	2.6	10.4	Steroids, MMF, CSA, ECP
22	85	14	IV	GI IV/Liver IV	4	1.4	5.5	Steroids, MMF, CSA, Everolimus
23	140	26	IV	GI IV/Liver IV	9	2.3	21.5	Steroids, MMF, CSA, Everolimus, ECP
24	21	73	IV	GI IV/Skin IV/Liver IV	3	1.5	3.5	Steroids, MMF, CSA, ECP
25	9	54	III	GI IV	2	4.4	8.8	Steroids, MMF, Infliximab, Tacrolimus
26	14	21	IV	Skin IV	1	4.0	4.0	Steroids, MMF, CSA

MSC: mesenchymal stromal cells; aGvHD: acute graft-versus-host disease; GI: gastrointestinal; CSA: cyclosporine A; MTX: methotrexate; MMF: mycophenolate mofetil; ECP: extracorporeal photopheresis; BW: body weight.

for chromosome 8q24 and that 3% of the MSCs displayed a tetraploid signal pattern (Figure 3E).

Comparison of the proliferation potential of MSC from individual donors, pooled MSCs from the 8 donors and MEP

Before MSC bank generation, we tested the capacity of BM-MNC from each donor to generate MSC. The number of generated MSCs per 1x10⁶ BM-MNCs after 13 days in culture varied by more than one order of magnitude, ranging from $0.5x10^5$ to $5.4x10^5$ MSC (Figure 4A).

Moreover, to validate the rationale of pooling BM-MNC from 8 donors to establish the MSC bank, we compared the *in vitro* proliferation capacity of the MSC from the 8 individual donors, the pooled MSC of the 8 individual donors, and the four MEP (Figure 4B). The MSC from each bone marrow donor showed different proliferation rates; these varied from $3x10^5$ MSC (donor 7) to $1.7x10^6$ MSC (donor 5). The mean of proliferation of the MSC from the 8 donors was $1x10^6 \pm 5x10^5$ MSC, which correlated well with the number of expanded MSC generated from the pooled-MSC from the 8 donors ($1.06x10^6$ MSC). Interestingly, both values correlated very well with the mean number of MSC obtained from the expansion of four MSC bank aliquots within a passage ($1.09x10^6 \pm 1 \times 10^5$ MSC). These results confirmed our hypothesis that pooling BM-MNC enables the generation of an "arithmetic mean" of high- and low-proliferating MSC.

Because the MSC in our bank were generated from a pool of BM-MNC from 8 "3rd-party" donors, we were interested in the contribution of the BM-MNC from each donor to the MEP. Chimeric analysis *via* STR-PCR using a series of genetic markers demonstrated the distinct proportions of the MEP derived from the 8 donor samples (Figure 4C). In principle, the relative contribution of each donor sample to the MEP did not strictly correlate with the proliferation potential of the MSC generated from the individual donors (Figure 4A). In addition, donor proportion in the MEP did not correlate with the relative donor proportion in the initially pooled BM-MNCs, which were used as a source for generation of our MSC bank (*Online Supplementary Table S1*).

Safety of MEP in severe aGvHD

Twenty-six patients with severe aGvHD were enrolled in this compassionate use study (Table 1). They received a median of 2.2×10^6 MSCs per kg BW (range 0.9-4.4×10^6 MSC per kg BW in 24 patients). One patient received 6×10^6 MSCs per kg BW, and another patient received 7.7×10^6 MSCs per kg BW (Table 2). Overall, a median of 3 (range 1-9) MSC infusions were administered to each patient. Only 2 patients exhibited adverse effects to MSC infusion (one incident each of headache and nausea), which presumably may be attributed to the cryoprotectant.

Response to treatment with MSC and overall survival

Based on the assessment criteria on day 28 after the initial MSC infusion, 5 of 26 patients (19%) showed a complete response (CR), 15 of 26 patients (58%) a partial response (PR), 4 of 26 patients (15%) did not respond, and 2 of 26 patients (8%) died before day 28 and thus their response could not be evaluated. Overall response rate, defined as patients with CR or PR, was 20 of 26 patients (77%). Follow up for 15 months demonstrated an increase in the CR rate to 73.1% (19 of 26) and a decrease in the percentage of patients experiencing a PR to 11.5% (3 of 26). This treatment resulted in a 2-year overall survival (OS) estimate of $71 \pm 11\%$ for the entire patient cohort (n=26) (Figure 5A), indicating the safety of this treatment and suggesting its efficacy *in vivo*. In addition, cumulative incidence (CI) of non-relapse mortality (NRM) estimate at two years in our patients was $15 \pm 7\%$ (Figure 5B). All details concerning the responses to MSC treatment are presented in Table 3.

A total of 4 of 26 patients died due to non-relapse mortality (NRM). Two of these 4 patients died due to multi-organ failure based on progressive aGvHD. One of these 4 patients died because of cerebral thromboembolism of unknown origin, whereas the other patient died due to uncontrollable infection. Another 2 patients died due to relapse of their underlying leukemia.

Prognostic factors

Using Fisher's exact test or the Kruskal-Wallis test, we found that none of the clinical factors (*Online Supplementary Table S4*), including sex, age, diagnosis, donor, conditioning regimen, graft source, GvHD prophylaxis, type and number of drugs used in the initial treatment, severity of aGvHD, and time of aGvHD onset, correlated with the response to MSC treatment.

Discussion

In the GvHD-related clinical studies reported so far, patients were treated with MSC generated from individual donors after their culture in either serum-containing[13] or serum-free media.[18-21] However, donor-to-donor heterogeneity[27-29] and the lack of standardized manufacturing protocols may lead to inconsistent clinical results that cannot be compared. In addition, we have recently demonstrated for the first time at the clonal level the intra-donor heterogeneity of the allosuppressive potential of MSC. Interestingly, the net allosuppressive effect of MSC represented an "arithmetic mean" of the high and low allosuppressive clones composing the MSC population.[7] Given these findings, the selection of an appropriate donor with
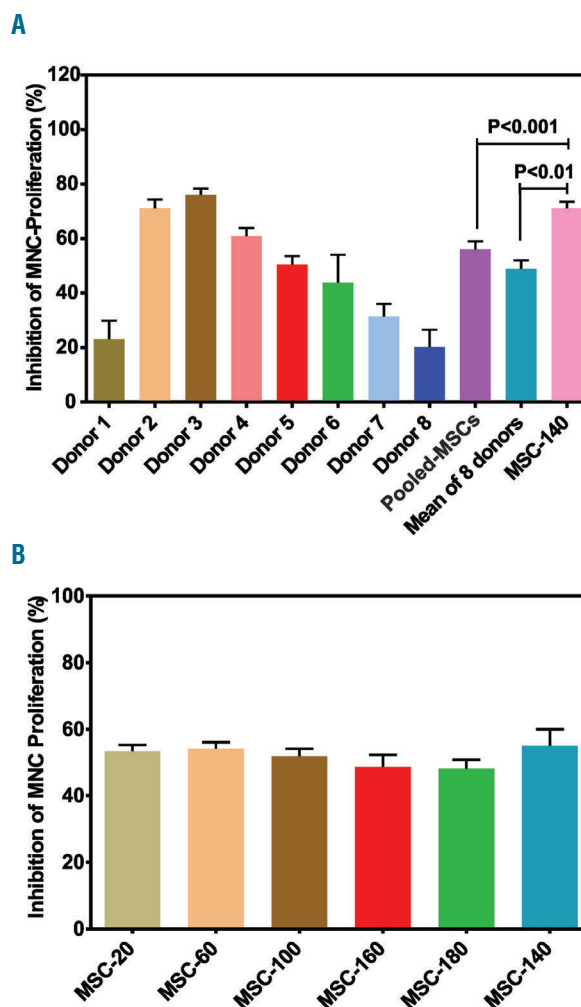
Figure 2. Allosuppressive potential of mesenchymal stromal cells (MSC) generated from individual donors and of MSC end-products (MEP). (A) MSC from the 8 individual donors and pooled MSC from these 8 donors (Pooled MSC); and one MEP (MSC-140) were expanded from the start of passage 2 to the end of passage 2. The MSC were evaluated for their allosuppressive effect in a mixed leukocyte reaction (MLR). (B) Six MEP (clinical doses) were thawed, washed, and directly tested in an MLR. Results presented as mean±SEM. Statistical analysis was performed using Student's *t*-test.

potent MSC is challenging and, so far, those in the scientific community who are studying MSC have not offered any solution. In an attempt to resolve this issue, in this study, we developed a novel strategy to circumvent or at least to minimize donor-to-donor heterogeneity by establishing an MSC bank from pooled BM-MNCs of multiple "3rd-party" donors (in our case, 8 donors) for the generation of clinical-scale MSC. To validate the rationale of this approach, we tested the allosuppressive effect of MEP from the MSC bank and of MSC derived from 8 donors individually. As expected, the allosuppressive potential of individual MSC was highly heterogeneous. MSC derived from the individuals displayed effects on the alloantigen-induced proliferation of PB-MNC ranging from 20% (donors 1 and 8) to approximately 80% inhibition (donors 2 and 3), with a mean allosuppressive effect of 48%. These results correlated very well with the strikingly high inter-donor differences in the immunosuppressive effects of MSC (range 0-90%).[30] In MLR, the allosuppressive

Table 3. Response to the mesenchymal stromal cell treatment.

Patient	At initial MSC treatment		Response at 28 days treatment after the initial MSC			Response at last follow up		Outcome	Follow up (months)
	aGvHD overall grade	aGvHD Involved organs and stage	aGvHD overall grade	aGvHD Involved organs and stage	Response	aGvHD overall grade	Response		
1	IV	GI IV/Skin IV/Liver IV	III	GI II/Liver II	PR	0	CR	Alive, cGvHD	9
2	III	GI III/Skin III	II	GI I/Skin I	PR	0	CR	Alive, cGvHD	10
3	III	GI IV	III	GI IV	NR	IV	NR	Alive	22
4	IV	Skin IV	*	*	death	I	PR**	Death (Thromboembolism)	1
5	IV	GI IV/Skin IV/Liver II	I	Skin I	PR	0	CR	Alive	39
6	III	GI III/Skin III/Liver III	II	GI II	PR	0	CR	Alive	13
7	IV	GI IV/Skin IV/Eyes IV	III	GI II/Skin II	PR	0	CR	Alive	10
8	II	GI I-II	I	Skin I	PR	0	CR**	Death (Relapse)	12
9	IV	GI III/Liver III	0	no	CR	III	PR**	Death (Relapse)	15
10	III	GI III	0	no	CR	0	CR	Alive, cGvHD	16
11	III	GI III/Skin III/Eyes III	II	GI II/Skin II	PR	0	CR	Alive	6
12	III	GI II/Skin II	0	no	CR	0	CR**	Death (Infection)	2
13	III	GI III/Skin III	0	no	CR	0	CR	Alive, cGvHD	24
14	III	GI IV	III	GI IV	NR	0	CR	Alive	42
15	IV	GI IV/Skin IV	II	GI I/Skin II	PR	0	CR	Alive	28
16	IV	GI IV/Skin II/Liver IV	III	GI II/Liver I	PR	0	CR	Alive	4
17	IV	GI IV/Skin IV	*	*	death	IV	NR**	Death (GvHD)	0.4
18	IV	GI IV/Skin IV	II	GI I/Skin II	PR	II	PR	Alive	11
19	IV	GI IV/Skin IV/Liver IV	I	Skin II	PR	0	CR	Alive	13
20	III	GI III	II	GI I	PR	0	CR	Alive	5
21	III	GI IV	III	GI IV	NR	0	CR	Alive	24
22	IV	GI IV/Liver IV	III	GI II/Liver II	PR	0	CR	Alive	6
23	IV	GI IV/Liver IV	III	GI II/Liver II	PR	IV	NR**	Death (GvHD)	2
24	IV	GI IV/Skin IV/Liver IV	II	GI III	PR	0	CR	Alive	20
25	III	GI IV	III	GI IV	NR	IV	NR	Alive	18
26	IV	Skin IV	0	no	CR	0	CR	Alive	25

*aGvHD: acute graft-versus-host disease; MSC: mesenchymal stromal cells; GI: gastrointestinal; CR: complete response; PR: partial response; NR: non-response; cGvHD: chronic graft-versus-host disease. *Death prior to day 28. **Last response status at follow up before the death of the patient.*

potential of pooled MSC from the 8 donors was similar to the mean allosuppressive potential of the MSC from each individual donor. Remarkably, the allosuppressive potential of the expanded MEP, generated from pooled BM-MNC, was significantly higher than that of the pooled MSC and the mean allosuppressive potential of the MSC from each individual donor. Therefore, generation of MSC from pooled BM-MNC of multiple donors appears to be more efficient than pooling MSC from several donors, which was reported to generate greater and more stable suppression *in vitro* and *in vivo*.[10,31,32] Importantly, all tested MEP demonstrated an equivalent allosuppressive effect *in vitro* after thawing (equipotent MSC doses) (mean 52±8.7%).

Although MSC banks provide a large number of "off-the-shelf" products, a few reports have cautioned that freeze-thawed MSC display lower therapeutic efficacy than fresh MSC.[3,33,34] In contrast, other studies[10,35-37] have demonstrated that cryopreserved MSC exhibit equivalent viability and immunosuppressive potential to freshly isolated MSC from cell culture. Consistent with these find-

ings, we found that freeze-thawed MEP displayed a viability of 95% and retained the ability to effectively suppress lymphocyte proliferation *in vitro*.

One of the major criteria required for the clinical application of MSC is that the MSC should enter senescence without undergoing oncogenic transformation. Tarte *et al.*[38] demonstrated that all bone marrow-derived MSC exhibit complete growth arrest at a PD between 35 and 52 and lack post-senescence proliferation even after long-term culture. Three of the thawed MEP, which were expanded for 13 passages, entered replicative senescence between passage 10 or 11 (30.2 CPD) after 68 days in culture. Their senescence was followed by increased levels of the cell cycle regulators p21 and p16 but no change in the TP53 gene expression. This finding is consistent with the data reported for lethally irradiated MSC.[39] Importantly, none of the three MEP expressed the proto-oncogene c-myc or hTERT, and no post-senescence proliferation was observed, as previously demonstrated in other studies.[28,40]

After thorough phenotypic, genetic and functional characterization of our MSC bank, we administered a total of
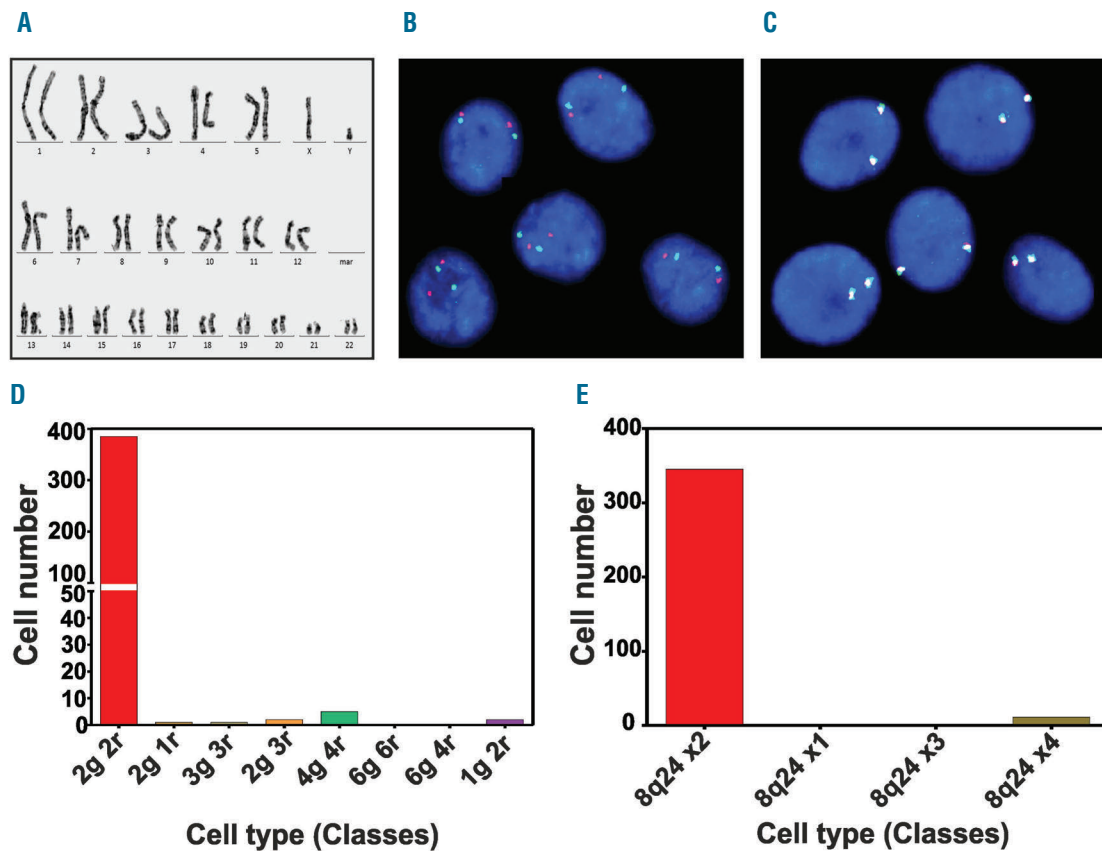
Figure 3. Genetic characterization of the clinical-grade mesenchymal stromal cells end-products (MEP). (A) Normal karyogram of MEP. (B) Interphase nuclei after 2-color hybridization of probe sets 5p15 (green) and 5q35 (red). (C) Interphase nuclei after 3-color hybridization of an MYC break-apart probe showed that almost all cells exhibited two normal fusion signals. (D) The number of MSC displaying a normal diploid or aneuploid pattern after 2-color hybridization of probe sets 5p15 and 5q35. (E) The number of MSC displaying a normal diploid or aneuploid pattern after 3-color hybridization of an MYC break-apart probe for chromosome 8q24.

81 MEP to 26 patients with severe refractory aGvHD on a compassionate use basis after individual approval by the regulatory authorities. All patients who received MSC infusions had exhibited failed responses to several other lines of treatment. It is known that the more drugs that fail in the treatment of aGvHD patients, the higher the risk that the patients succumb to GvHD. We did not observe any MSC-related side-effects during transfusion. However, 2 patients died due to progressive GvHD, 2 patients developed a relapse of their underlying leukemia, and 2 others died due to treatment complications. One of these died from infectious complications; as this patient was heavily immunosuppressed at the time of MSC infusion, it is impossible to attribute this event to any single treatment. As the relapse rate in our patients with malignant disease was only 9%, we found no evidence that MSC might hamper a graft-*versus*-leukemia effect.

In our cohort, 20 of 26 patients (77%) responded to the MSC treatment by day 28 (overall response), which is comparable to the results obtained in a randomized placebo-controlled study by using MSC product from the Osiris company (Prochymal) for the treatment of aGvHD (63%). Although the primary end point in that study was not achieved for the whole group of patients, there was a significant benefit over placebo group in the liver and GI tract.[41] Response rate in our cohort of patients is also similar to that reported by Introna *et al.*[20] in a cohort of 12 pediatric patients; that study demonstrated an overall response

of 66.7%. However, in their patient cohort, only 25% of the patients exhibited aGvHD over grade III, whereas in our series, 96% of the patients exhibited aGvHD grade III or IV. Lucchini *et al.*[19] observed a 62.5% overall response among 8 patients with aGvHD (50% grade I/II and 50% grade III/IV). Similar findings were reported by Prasad *et al.*[14] in a compassionate use study (overall response of 66.7% at day 32 in 12 pediatric patients) and by Kurtzberg *et al.*[15] (61.3% overall response in a large cohort of 75 pediatric patients after treatment of aGvHD with Prochymal). Our results are comparable to the results of the latter group considering the composition of our patient cohort (96% grade III/IV and only 4% grade II), which represents a very challenging patient population.

The CR rate increased from 19% at day 28 to 73.1% at the last follow up, whereas the primary PR rate decreased from 58% to 11.5%, indicating that tissue recovery requires time. The inversion of PR by the time in CR is similar to the data obtained by Le Blanc[46] in their pediatric cohort. The increased CR rate in our cohort translated into a very favorable 2-year OS estimate of 71%. Kurtzberg *et al.*[15] observed an OS of 57.3% (43 out of 75 patients) at day 100, and this value was similar to the OS rate at two years (40%) in a study by Prasad *et al.*[14] and Le Blanc *et al.*[42] who observed an OS rate at two years of 45% in their cohort of pediatric patients. Although our study was not randomized, considering that our patients were treated in very advanced stages of aGvHD the survival rate is very
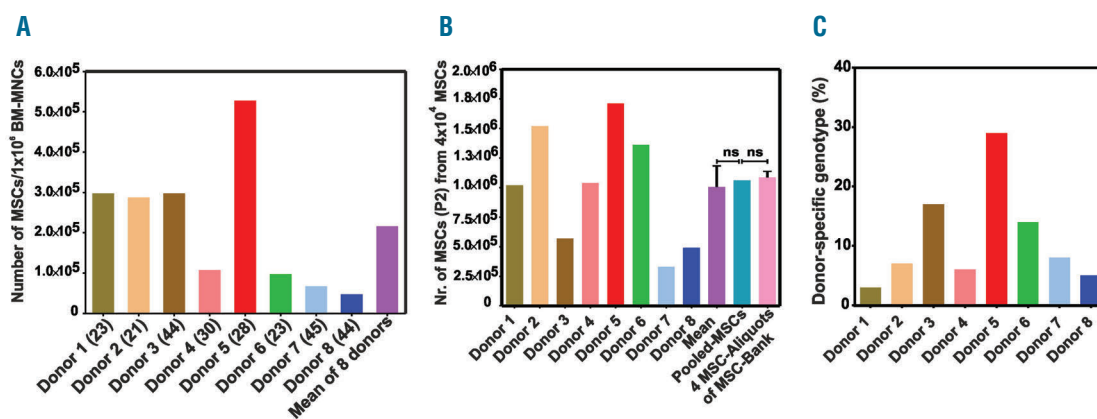
Figure 4. Capacity of bone marrow mononuclear cells (BM-MNC) from the 8 donors to generate mesenchymal stromal cells (MSC), their proliferation potential and chimeric analysis of the MSC end-products (MEP). (A) Data are expressed as the number of generated MSC per 1×10^6 BM-MNC from each donor cultured in 5% human platelet lysis (PL) for 13 days. Age of each donor (in years) is shown in brackets. (B) Comparison of the proliferation potential of MSC from the 8 individual donors with that of pooled MSC from the 8 individual donors and four MEP. (C) Determination of the relative proportion of each donor in MEP *via* STR-PCR; ns: not significant. Statistical analysis was performed using Student's *t*-test.
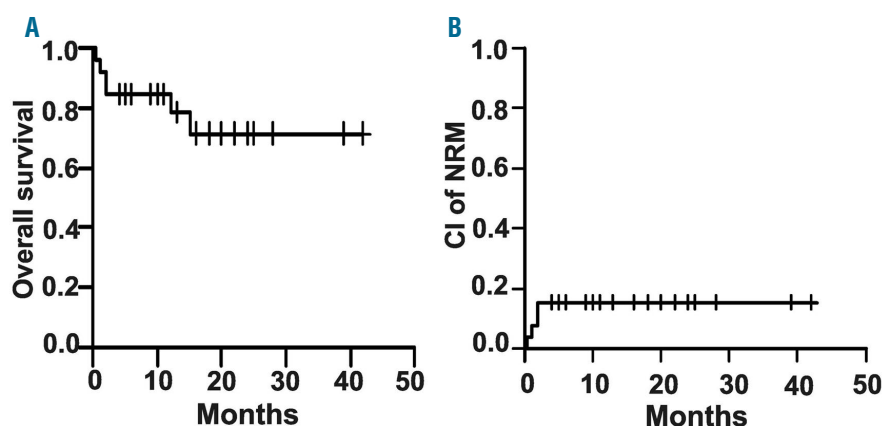
Figure 5. Overall survival (OS) and non-relapse mortality (NRM). (A) For all patients (n=26), OS estimate at two years was 71%±11%. (B) Cumulative incidence (CI) of NRM estimate at two years was 15%±7%. Estimates presented as mean±SE.

encouraging (*Online Supplementary Table S5*).

In summary, to our knowledge this is the first serum-free MSC bank generated from pooled BM-MNC of multiple donors as a source for bulk production of clinical-grade MSC with a predictable potency. Importantly, clinical data presented in this study demonstrated the *in vivo* safety and efficacy of MEP. Although the results of this single patient treatment are encouraging, a prospective randomized study is required to evaluate the beneficial effect of MEP as a novel cell-based therapy in the treatment of severe aGvHD.

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