Reduction of Graft-versus-Host-Disease in NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) Mice by Cotransplantation of Syngeneic Human Umbilical Cord-Derived Mesenchymal Stromal Cells

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ABSTRACT

Graft-versus-host disease (GVHD) is one of the major complications following hematopoietic stem cell transplantation, which remains the sole curative therapy for many malignant diseases of the hematopoietic system. The immunomodulatory potential of mesenchymal stromal cells (MSCs) to treat GVHD is currently being tested in various preclinical and clinical trials. Because the results of the preclinical and clinical trials on the use of MSCs to treat GVHD have not been consistent, we analyzed the potential beneficial effects of syngeneic versus allogeneic treatment, culture expansion of MSCs, and various MSC cell doses and time points of MSC transplantation in a murine GVHD model. We established the murine GVHD model based on the transplantation of umbilical cord blood-derived hematopoietic stem cells (UC-HSCs) and used this model to assess the therapeutic potential of umbilical cord blood-derived MSCs (UC-MSCs). The use of HSC and MSC populations derived from the same donor allowed us to exclude third-party cells and test the UC-HSCs and UC-MSCs in a matched setting. Moreover, we were able to compare various doses, transplantation time points, and the influence of culture expansion of MSCs on the impact of treatment. This resulted in 16 different treatment groups. The most efficient setting for treatment of UC-HSC-induced GVHD reactions was based on the simultaneous administration of $1 \times 10^6$ culture-expanded, syngeneically matched UC-MSCs. This therapy effectively reduced the number of CD8+ T cells in the blood, protected the mice from weight loss, and prolonged their survival until the end of observation period. Taken together, our data show beneficial effects of (1) syngeneic over allogeneic UC-HSCs and UC-MSCs, (2) culture-expanded cells over freshly isolated primary cells, (3) simultaneous over sequential administration, and (4) high doses of UC-MSCs. The animal model of GVHD established here is now available for more detailed studies, including a comparative analysis of the efficacy of MSCs derived from alternative sources, such as adipose tissue and bone marrow.

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) remains the sole curative therapy for many hematologic disorders [1]; however, the establishment of donor-derived hematopoiesis is often accompanied by graft-versus-host disease (GVHD). In contrast to the graft-versus-leukemia effect, in which donor-derived immune cells attack remaining malignant cells [2], GVHD is characterized by a donor-derived immune cell attack on host tissues [3]. The risk of developing GVHD increases with the degree of mismatch in histocompatibility antigens, but the condition still develops in up to 40% of HLA-matched cases [4]. The conventional prophylaxis and therapy for GVHD uses the immunosuppressants cyclosporin A, methotrexate, and corticosteroids [5], each of which can result in severe side effects arising either from global suppression of the acquired immune system or from damage to the liver, kidney, and gut caused by metabolized drug products [6]. Furthermore, a significant proportion of patients with GVHD develop a steroid-refractory condition for which there are limited therapeutic options.

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One strategy being increasingly used in such cases is the infusion of in vitro-expanded mesenchymal stromal cells (MSCs) [7]. MSCs are known to be immune-privileged and to ameliorate excessive immune reactions [8,9]. Although the precise mechanism of action in vivo remains unclear [10], the beneficial effect on GVHD is thought to involve MSC-derived trophic factors (including prostaglandin E2, indoleamine 2,3-dioxygenase 2, and nitric oxide) that can suppress B, T, and natural killer (NK) cells, while stimulating regulatory cells (reviewed in [11]). The immunomodulatory function of MSCs appears to be influenced by several variables, including their differentiation state [12,13], culture setting, cell density [14,15], and time in culture [16]. Moreover, recently reported data show that exosomes produced by MSCs derived from adipose or umbilical cord (UC) tissue have protein profiles suggestive of source-specific differences in immunomodulatory properties [17].

Although most of the work to date has involved MSCs derived from bone marrow, comparable cells can be isolated from various tissues, including UC tissue [18,19]. Because this neonatal tissue is readily available without the need for an invasive procedure [20], the ethical barriers for its use in cell therapy are relatively low [21]. Neonatal mesenchymal tissue has a high content of nonhematopoietic, young, and highly proliferative cells with high differentiation potential [22]. Furthermore, the high yield per cord reduces the number of population doublings needed to generate a therapeutic dose [23]. Thus, for the development of a xenogeneic GVHD MSC transplantation mouse model, the extraembryonic tissues offer a potential source of both therapeutic MSCs and hematopoietic stem cells (HSCs), making it feasible to collect and transplant genetically identical hematopoietic and mesenchymal populations from the same readily accessible tissue. Despite the lower numbers of HSCs in umbilical cord blood (UCB) [24] and the decline in the use of UCB as the practice of haploidentical transplantation becomes more established [25], UCB can still be indicated when no suitable haploidentical donor is available. This is most relevant for patients of non-western European ancestry, for whom UCB HSCT significantly improves access to an allologeneic transplant regardless of ethnicity [26,27]. However, even in a recent trial of Caucasian patients in which haploidentical transplants were reported to have superior outcomes compared with UCB transplants, 15% of the patients randomized into the haploidentical arm had to be crossed over into the UCB arm owing to the lack of a suitable donor [28]. Although UCB continues to be a valid transplant source, a deeper understanding of the variables influencing outcome following UCB transplantation has the potential to improve current outcomes.

One of the perceived benefits of UCB-derived HSCs for transplantation is that the severity of GVHD tends to be lower [30], because T cells in the UCB are naïve compared with those collected from adult peripheral blood (PB) by apheresis [31]. However, even with the use of UCB, GVHD remains a severe clinical problem and the main complication after HSCT [32]. The hypothesis investigated here is that the simultaneous or sequential cotransplantation of UC-MSCs decreases or prevents the development of acute GVHD. The main aims of this study were first to establish a NOD.Cg-Pkdcdicead I2purgmtw1Sqd (NSG) mouse model in which T cell-initiated GVHD is induced by human UCB-derived mononuclear cells (UCB-MNCs) and then to assess the ability of primary or cultured UC-MSCs derived from syngeneic or allogeneic donors to alleviate the GVH reaction.

METHODS
Tissue and Cell Sources, Culture, and Maintenance of MSCs
Human UC and UCB were collected at the St Elisabeth and St Barbara Hospital (Halle, Germany) after obtaining a signed declaration of informed consent from each expectant mothers. Approval was obtained from the local Ethics Committee (reference nos. 364-13-16122013 and 115-11-18042011). All samples were used anonymously. After collection of UCB, 10 to 50 cm of the UC was aseptically transferred into sterile transportation vessels containing 0.9% sterile NaCl solution (Serumwerk Bernburg, Bernburg, Germany) supplemented with 10 mg/mL clindamycin (Ratspharm, Ulm, Germany) and 50 mg/L gentamicin (K. Braun, Melsungen, Germany). After transport to the laboratory within 24 h, the UCs were washed repeatedly in NaCl solution and Prontosan (B. Braun). A scalpel was used to cut the UC into 5-mm-thick slices. Cell isolation was achieved by incubating the tissue slices with collagenase, hyaluronidase (both from Serva, Heidelberg, Germany), and dispase II (Sigma-Aldrich, St Louis, MO). We used a 2-step dissociation procedure, starting with a 12-hour static cooling period (4 °C) and followed by a 6-hour heating period (37 °C) under constant rotary mixing. After enzymatic digestion, cells were centrifuged at 300 × g for 5 minutes and then washed with 1× PBS (Lonza, Basel, Switzerland). A sample was used for flow cytometry counting of nucleated and viable cells following staining with 100 ng/mL acridine orange and 2 μg/mL propidium iodide (both from Sigma-Aldrich) for 10 minutes. For standard in vitro expansion, the cells were seeded to tissue culture-treated plasticware (Greiner Bio One, Kremsmunster, Austria) at a density of 6000 cells/cm² in αMEM (Lonza) supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine (PAA, Pasching, Austria), and 5% to 10% selected FBS (Biochrom, Berlin, Germany). The medium was changed 2 to 3 times a week, and cells were subcultured 1:10 when reaching 80% to 90% confluence. Application of Accumax (Sigma-Aldrich) for 5 min was used for detection of cells. For cryopreservation, ProFreeze (PAA) was used according to the manufacturer’s instructions. Consistent with our previous findings [33], all batches of UC-MSCs were capable of adipogetic and osteogenetic differentiation in standardized differentiation assays (data not shown).

UCB-MNC Isolation
UCB was either diluted 1:2 with 1× PBS and loaded over 1.077 g/mL Biocoll (for standard density gradient centrifugation according to the manufacturer’s instructions) or supplemented with 7.5% dimethyl sulfoxide (Serumwerk Bernburg) and cryopreserved using a computer-controlled rate freezer (IceCube 14M; Sy-Lab Gerate, Neupokurndorf, Austria). Cryopreserved UCB was thawed, and MNCs were purified by density gradient centrifugation directly before setting up the experiments. UCB-MNC preparations were cryopreserved using ProFreeze according to the manufacturer’s instructions.

Flow Cytometry Analysis of UCB-MSC
For surface marker analysis, UCB-MSCs were examined directly after isolation as well as at passages 1, 2, and 4. The cells (1 × 10⁹ per sample) were resuspended in 100 µL of 1× PBS for flow cytometry analysis and stained with 1 µg/mL PE-cy5.5-conjugated (CD105, CD19, CD166, CD90, CD73) and 10% selected FBS (Biochrom, Berlin, Germany). The medium was exchanged 2 to 3 times a week, and cells were subcultured 1:10 when reaching 80% to 90% confluence. Application of Accumax (Sigma-Aldrich) for 5 min was used for detection of cells. For cryopreservation, ProFreeze (PAA) was used according to the manufacturer’s instructions.

Colony-Formation Assays
For limiting dilution assay of CFU-f (colony forming units-fibroblast) frequency, the cells were seeded into 96-well plates at 5000 cells/well and cultured with standard culture medium supplemented with 2 ng/mL basic fibroblast growth factor (FGF-2; PeproTech, Hamburg, Germany) at densities of 1, 3, 9, and 27 cells per well (serial dilution). After a 14-day incubation under low oxygen (3% O₂), the colonies were stained with 0.1% crystal violet (Carl Roth, Karlsruhe, Germany), and wells containing colonies (>20 cells) were counted manually. L-Calc software (StemCell Technologies, Cologne, Germany) was used to calculate the CFU-f frequency.

Mixed-Lymphocyte Reactions
PB samples were used both fresh and following cryopreservation and thawing, whereas UC samples were used exclusively as fresh-thawed, cryopreserved cells. MNCs were isolated by density centrifugation using Biocoll with a density of 1.077 g/mL. Single PB mononuclear cell (PBMC) samples were in multiple mixed-lymphocyte reactions (MLRs) to limit the donor variability. MLRs were set up in triplicate in flat-bottomed 96-well plates with 10⁶ responder and 10⁵ irradiated stimulator cells (30 Gy) each in MLR medium consisting of RPMI-1640 (Biochrom), 5% human AB serum (Sigma-Aldrich), and 1% Pen/Strep solution (PAA). Wells containing either stimulator or responder cells served as controls. After 6 days of culture, 1 µCi ³H-thymidine (Amersham, Little Chalfont, UK) was added to each well. After another 16 hours (on day 7), the incorporation of labeled thymidine as a measure for cell proliferation was monitored using a scintillation counter (PerkinElmer, Waltham, MA).

Cytokine-primed MLRs were started by incubating stimulator cells in MLR medium supplemented with 18 U IL-2/mL and 500 U TNF-α/mL (both from PeproTech, Rocky Hill, NJ) for 3 days. Stimulator cells were then harvested, washed, irradiated with 30 Gy, and pooled with responder cells in
MLR medium supplemented with 1000 U/IL-4/mL and 1000 U IFN-γ/mL (both from PeproTech). Following a 6-day culture, 3H-thymidine was incorporated into cells in MLR medium supplemented with 1000 U IL-4/mL and 1000 U IFN-γ/mL (NSC) mice were purchased at age 6 to 7 weeks from Charles River Germany (Sulzfeld, Germany). All mice were housed, treated, and handled in accordance with the guidelines of the University of Leipzig Animal Care Committee and the Regional Board of Animal Care for Leipzig.

**Transplantation**

Before transplantation, all mice were conditioned by sublethal total body irradiation (3 Gy) on an X-ray apparatus (D3225 orthovoltage; Gullmay Medical, Cambridge, UK). Depending on group assignment, they received combinations of 150 µL of 1× PBS supplemented with 0.5% BSA (Serva), specific UCB-MNC counts (0.05 x 10^6, 1 x 10^6, 1.75 x 10^6, 2 x 10^6, or 10 x 10^6), and specific UC-MSC counts (0.05 x 10^6 or 1 x 10^6). The cells were thawed and washed with 1 × PBS at 2 to 3 h before transplantation and stored at room temperature until transplantation. Because we were interested primarily in the potential of UC-MSCs as an early intervention option applied either together with the HSCTs or at the first signs of GVHD, we chose the d0 and d12 to d14 time points for MSC treatment. The culture-expanded UC-MSCs used for transplantations were cultivated for 2 or 3 passages, equivalent to a maximum of 10 population doublings. The gays were transplanted by systemic application (i.e., injection into the lateral tail vein or intraperitoneal injection) of recipient mice. To generate true biological replicates, separate donors were used for each of the 6 animals receiving syngeneic matched MSCs and MNCs (1 donor per animal), whereas the same MSC preparations were used to transplant MNCs from another 6 donors for the allogeneic settings. The overall health status and event-free survival (EFS) of the mice were documented daily, including the determination of the whole body weight and the GVHD clinical score [34]. In addition, cell engraftment, as well as the hematologic and immunologic reconstitution of both cell and murine cells, were measured weekly by whole blood count and flow cytometry (see next section). Monitoring was carried out for up to 60 days after MNC transplantation.

**Flow Cytometry Analysis of MLR Assays and Murine Blood Samples**

The cells used for flow cytometry analysis included freshly isolated PB-MNCs, cells harvested from MLC assays, and freshly drawn murine blood samples with the addition of 50 U IL-hapten per sample (Roche Diagnostics, Rotkreuz, Switzerland). Murine blood samples (approximately 100 µL) were counted using an animal blood counter (abc Classic; scil animal care company, Viernheim, Germany) before antibody staining.

For flow cytometry staining, cell suspensions in 1 × PBS were added to 5 mL round-bottom tubes (BD, Franklin Lakes, NJ). Monoclonal antibodies were added according to the manufacturer’s instructions and incubated at room temperature for 20 minutes. For whole blood samples, 1 mL of 1 × BD Pharm Lyse lysing solution (BD Biosciences, diluted with deionized water according to the manufacturer's instructions) was added, followed by incubation for 10 min at room temperature in the dark. The cells were then washed twice with 1 mL of 1 × PBS, resuspended in 100 µL of 1 × PBS, and analyzed on either a FACSCanto II or a LSR II flow cytometer (both from BD Biosciences). The monoclonal antibodies used were coupled to fluorescent isothiocyanate (anti-human CD3, anti-human HLA-DR), PE (anti-human CD4, anti-human CD44, anti-human CD73, anti-human CD90, anti-human CD105, anti-human CD166), APC (anti-human CD19), PE-Cy7 (anti-human CD8), anti-human CD44, anti-human CD73, anti-human CD90, anti-human CD105, anti-human CD166, APC (anti-human CD19), PE-Cy7 (anti-human CD8), PerCP (anti-human CD14), APC-H7 (anti-human CD45), or APC-Cy7 (anti-murine CD45, respectively) (all from BD Biosciences). At least 10,000 events were recorded, and all events with fluorescence exceeding that in unstained controls were classified as positive. Data were exported as FCS files and analyzed using Flowjo software (version 10.1r5; Flowjo, Ashland, OR) or FACS Diva software (version 8.0.2; BD Biosciences).

**Quantitative Scoring of Results and Statistical Analysis**

R version 3.6.1 [35], including the packages ggfortify (version 0.4.7) [36,37], lemon (version 0.4.3) [38], xfun (version 0.10) [39], scales (version 1.0.0) [40], ggpubr (version 0.2.3) [41], magrittr (version 1.5) [42], tidyr (version 1.0.0) [43], ggplot2 (version 3.2.1) [44], survival (version 2.44-1.1) [45], car (version 2.0-3) [46], coin (version 1.3-1) [47], coxphf (version 0.1) [48], gsn (version 0.8.8) [49], were used for data processing, calculations, and visualization. Data in general are presented as mean ± SD unless indicated otherwise. Statistical significance was calculated using the Student or Welch t test, the asymptotic Wilcoxon-Mann-Whitney test (taking ties into account), or the log-rank test in the case of Kaplan-Meier estimators for cumulative survival curves and adjusted for multiple comparisons by the false discovery rate (FDR), where indicated.

For comparison of the overall beneficial effects of the various treatment strategies, an overall GVHD score was calculated from 4 subscores: (1) CD8+ events (% lymphocytes) from flow cytometry analysis, (2) clinical GVHD score, (3) normalized weights, and (4) EFS. Subscore 1 was calculated from the groupwise maximum value of a locally weighted scatterplot smoothing (LOWESS) estimator over the overall observation period. Because of deceased animals and different distributions between groups, this robust method was chosen to estimate a subscore indicative for the percentage of cytotoxic T cells irrespective of the time point at which the maximum value was reached. Subscores (2) and (3) were estimated from group-specific LOWESS estimators as well; however, unlike the percentage of CD8+ events in flow cytometry analysis that are related directly to human cells, animal body weights (normalized to the individual weight at the day of MNC transplantation) and clinical GVHD scores can be influenced by irradiation effects. Therefore, only the time span after the irradiation control group recovered was considered—that is, after the LOWESS estimator of the irradiation control group’s normalized body weights returned to 100% of the initial weight (d23) and after the LOWESS estimator or the irradiation control group’s clinical GVHD score started to decrease again (d20). Subscore (4) was calculated from the hazard ratios of a Cox proportional hazards model with treatment group affiliation as the sole factor. The respective subscores from the GVHD control group were set to 10, and those from the irradiation control group were set to 0, and the subscores of the treatment groups were calculated according to this scale. Subscores <0 or >10 were truncated. The sum of these normalized subscores represents the overall GVHD score with a maximum achievable value of 40.

A thorough EFS analysis using Cox proportional hazard models was performed with the GVHD control group 15 as the reference. The irradiation control group 0 was removed from the dataset for this purpose. First, each parameter (MSC dose, culture expansion, simultaneous or sequential transplantation, and genetic match of UCB-MNCs and MSCs) was considered separately. If the fit resulted in a P value ≤0.05, then the respective factor was considered for interaction analysis with the other statistically significant parameters.

We did not perform inferential statistics on the flow cytometry data owing to multiple parent populations when dealing with compositional data.

**RESULTS**

**Cells Isolated from UCs by Enzymatic Digestion Contain Precursors of MSCs**

MSCs were isolated from UCs by enzymatic digestion with a recovery of 2.74 x 10^6 ± 8.64 x 10^5 cells/g (n = 10). The colony-forming activity of freshly isolated cells in CFU-f assays was 14.9 ± 7.6% (n = 10). Freshly isolated cells (passage 0) and expanded cells (passages 1, 2, and 4) were subjected to flow cytometry analysis for surface markers characteristic of MSCs (Figure 1). From passage 2 onward, the cultured cells clearly exhibited the classic surface profile of MSCs, with >90% of all cells expressing CD44, CD73, CD90, CD105, and CD166 (Figure 1A) and low (<2%) or absent expression of hematopoietic markers CD45, CD3, CD19, CD34, and HLA-DR (Figure 1B). The freshly isolated cells (passage 0 in Figure 1) were more heterogeneous, with 91.85 ± 4.49% expressing CD90 but only 40.48 ± 7.88% expressing CD73 and a minority of cells expressing the other markers characteristic of cultured MSCs. The low frequencies of hematopoietic cells present in the freshly isolated population (8.2 ± 6.3% CD3+ cells) were rapidly depleted during culture.

**Cytokine-Primed UCB-MNCs Are Active in MLRs**

To explore the potential of UC-MSCs for treating GVHD arising from UCB-MNCs, we first established an in vitro test to determine the proliferative potential of UCB-MNCs. For this purpose, MLRs were set up using UCB-MNCs from different donors as responder and stimulator cells. PBMCs served as a positive control. The proliferation response was assessed by 3H-thymidine incorporation and by flow cytometry for T lymphocyte markers. Unstimulated UCB-MNCs generated a markedly lower proliferative response than that of the PBMC control (Figure 2C and D); however, priming of the stimulator cells with TNF-α and IL-2 before irradiation and the addition of IL-4 and IFN-γ during MLRs (Figure 2A) resulted in a proliferative response (Figure 2C). This finding confirms the
proliferation competence of cryopreserved UCB-MNCs. Flow cytometry analysis of UCB MLRs (Figure 2B) showed an increase in CD3+CD8+ cytotoxic T cells during the assay, although the intensity of CD8 positivity per cell was reduced somewhat compared with that in the d0 population (data not shown). There were no extensive alterations in CD3+CD4+ T helper cells or overall T cells (CD3+), indicating that the proliferative response in UCB-MNC MLR reactions is based mainly on CD8+ cell proliferation.

**Naïve UCB-MNCs Can Induce a Clinically Significant GVHD-Like Reaction in Vivo**

To assess the potential of UC-MSCs to suppress a GVHD-like reaction in vivo, a murine model of GVHD was established (also compare [28]). Both cryopreserved UCB-MNCs originally prepared from fresh whole blood and fresh UCB-MNCs prepared from cryopreserved whole blood were administered to irradiated NSG mice at various cell doses (0 to 10 × 10⁶ UCB-MNCs). The distinct GVHD induction groups and the corresponding median survival times are listed in Table 1.

A GVHD induction group was selected based on a robust disease manifestation and progression while maintaining a moderate survival. Transplantation of 1 × 10⁷ UCB-MNCs led to median survival times of <2 weeks regardless of whether the MNCs were prepared directly from cryopreserved whole blood (group 4) or prepared from fresh blood and then cryopreserved (group 2). At the other extreme, the administration of 5 × 10⁵ cells could not induce a robust GVHD based on disease monitoring (data not shown). However, transplantation of 1.75 × 10⁶ MNCs freshly prepared from cryopreserved UCB satisfied the criteria by stimulating robust GVHD symptoms. Human cells engrafted in the recipient mice and contributed to leukocyte and T cell subsets (Figure 3A). There was a rapid increase in the numbers of CD8+ cytotoxic T cells from d19 onward (Figure 3B) (d19, 0.8%; d33, 7.3%; d47, 9.7%), a clinical GVHD score
of up to 6, and a weight loss of >15% of initial weight. Daywise comparisons were made when data from at least 3 animals per group were available, but these comparisons did not reveal statistically significant results after FDR adjustment (Welch 2-sample t test for normalized weights, asymptotic Wilcoxon-Mann-Whitney test for clinical GVHD scores). The median EFS was 31 days (Figure 3B; cumulative survival, blue curve), which was statistically significantly shorter than that of the control group receiving irradiation only (3 Gy) (PEFS = .0186, log-rank test).

**Table 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>MNCs, × 10⁶</th>
<th>Cryopreserved as</th>
<th>No.</th>
<th>Irradiation</th>
<th>Application Time after Irradiation, h</th>
<th>Median Survival Time, d</th>
<th>Description</th>
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</thead>
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<tr>
<td>0</td>
<td>0.5</td>
<td>UCB-MNCs</td>
<td>4</td>
<td>3 Gy</td>
<td>2-3</td>
<td>NA</td>
<td>Irradiation control</td>
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<tr>
<td>1</td>
<td>10</td>
<td>Full UCB</td>
<td>5</td>
<td>3 Gy</td>
<td>2-3</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>Full UCB</td>
<td>6</td>
<td>3 Gy</td>
<td>2-3</td>
<td>15.5</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>Full UCB</td>
<td>5</td>
<td>3 Gy</td>
<td>2-3</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>Full UCB</td>
<td>5</td>
<td>3 Gy</td>
<td>2-3</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>Full UCB</td>
<td>6</td>
<td>3 Gy</td>
<td>2-3</td>
<td>31</td>
<td></td>
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</tbody>
</table>

NA indicates not applicable (ie, 0, 1, or 2 events).

**UC-MSCs Are Able to Impede a GVHD-Like Reaction in Vivo**

The NSG mouse model described above was used to test the ability of infused UC-MSCs to suppress the GVHD reaction in vivo. A total of 16 different treatment groups were set up (Table 2), with differences in 4 variables: MSC amount (5 × 10⁴ or 1 × 10⁶ per mouse), culture (freshly isolated or expanded), simultaneous UCB-MNC and UC-MSC versus sequential transplantation (UC-MSCs 12 to 14 days after UBC-MNCs), and matched (syngeneic) versus mismatched (allogeneic) UCB-MNC and UC-MSC transplantation.
All mice were followed for up to 60 days after UCB-MNC infusion, with regular assessment of survival, clinical GVHD score, weight, and human CD45+, CD3+, and CD8+ events in PB. An overall GVHD score calculated from these parameters and the hazard ratios of the EFS rates summarizes the degree of GVHD suppression in each experimental group (Figure 4A). Detailed results for the group showing the clearest suppression of GVHD (ie, the treatment group having the lowest overall GVHD score) are shown in Figure 4B. This group (group 16) was treated with $1 \times 10^6$ syngeneic, culture-expanded UC-MSCs. All mice from this group survived until the end of the experiment and showed reduced percentages of CD45+ cells, CD3+ T cells, and CD8+ cytotoxic T cells compared with the control group receiving only MNCs, together with a weight curve and GVHD scores comparable to those of the irradiation control group (Figure 4B). However, daily comparisons of normalized weights were not statistically significantly different from those in GVHD control group 15 after FDR adjustment (Welch 2-sample $t$ test for normalized weights), whereas the FDR-adjusted asymptotic Wilcoxon-Mann-Whitney test for differences in the clinical GVHD score showed statistically significantly lower clinical GVHD scores on d5, d6, d13, d14, d20, d27, d28, d34, and d35 compared with the GVHD control group (Figure 4B).

The thorough exploratory EFS analysis using a Cox proportional hazard model revealed a positive influence of the following factors compared with the GVHD control group: use of culture-expanded cells ($P = .08$) over freshly isolated cells ($P = .10$), simultaneous ($P = .03$) over sequential transplantation ($P = .20$), syngeneic ($P = .02$) over allogeneic match of UCB-MNCs and UC-MSCs ($P = .28$), and low-dose MSCs ($P = .05$) over high-dose MSCs ($P = .16$). For interaction analysis, expansion of MSCs was not considered, because the fit did not show a $P$ value $< .05$. The groups receiving MSCs in a cotransplantation with syngeneic MNCs showed statistically significantly better EFS ($P_{1e6\text{MSCS}} = .03$, $P_{5e4\text{MSCS}} = .01$). Because the possibility that differences between MSC counts were caused by the smaller group size receiving $1 \times 10^6$ MSCs cannot be
excluded, this factor probably was not as important as suggested from the previous one-factor analysis. The final model had an overall $P$ value of .03.

### DISCUSSION

Following HSCT to cure leukemia, a low-level activation of donor-derived immune cells against host tissues may provide a survival advantage by eliminating remnant disease through the graft-versus-leukemia effect [51]. However, if this immune reaction extends to normal host tissues, as occurs in up to 40% of allogeneic HSCT recipients [4], the ensuing GVHD can become seriously debilitating and life-threatening, given the limited available treatment options. Thus, there is a need for new and effective strategies to control the degree and intensity of graft-mediated immune reactions.

MSCs offer a promising resource in this respect, with their ability of MSCs to suppress GVHD in a clinical setting (see [56]). However, uncertainty remains about their precise mode of action and the variables, such as source tissue, culture conditions, dose, and time of application, that may influence the ability of MSCs to suppress GVHD in a clinical setting (see [56] and [57] for recent reviews).

Postnatal UC offers some potential advantages as a source for MSCs, being widely available, easily accessible, and rich in MSCs compared with bone marrow and adipose tissue [22,58]. In theory, the use of UC-MSCs also would offer the novel possibility of using both HSCs (for reconstitution) and MSCs (for immune modulation) from the same donor, thereby avoiding the need for third-party cells. Whether this simplification of the immune environment would provide an advantage to the patient is unclear, but given the uncertainties surrounding the modes of MSC action and determinants of response, this issue should be considered.

The aim of the present study was therefore to explore the potential of UC-derived MSCs to suppress a GVHD-like immune reaction. Our initial tests confirmed that the UC-MSCs used in this study carried the characteristic MSC surface markers. However, because an MLR is at best an indirect indicator of in vivo potency [10], we used a xenogeneic animal model in which human UCB-MNCs were used to induce a GVHD-like disease in immunocompromised mice. Using conditions under which 5 of 6 control mice met the termination criteria of GVHD-like disease, we found that UC-MSCs were indeed capable of ameliorating the typical disease symptoms and markedly increasing EFS. The clearest combined effects (resulting in EFS in 6 of 6 mice, body weight recovery, a maximum clinical GVHD score of 1, and lower percentages of CD8+ events indicative of cytotoxic T cells in the peritoneal washings) were achieved by transfusing a large number ($1 \times 10^6$ per mouse) of culture-expanded, syngeneic MSCs at the same time as the UCB-MNCs.

The most important single factor for a longer EFS was the transplantation of syngeneic rather than allogeneic MSCs,

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
<th>UCB-MNCs, $\times 10^6$</th>
<th>No.</th>
<th>Irradiation</th>
<th>MSC Application Time after Irradiation</th>
<th>UC-MSC Culture</th>
<th>MSC Dose, $\times 10^6$</th>
<th>Genetics</th>
<th>Median Survival, d</th>
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<tbody>
<tr>
<td>7</td>
<td>High MSCs, fresh, syngeneic; d0</td>
<td>1.75</td>
<td>4</td>
<td>3 Gy</td>
<td>2-3 h</td>
<td>Freshly isolated</td>
<td>1</td>
<td>Syngeneic</td>
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</tr>
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<td>8</td>
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<td>1.75</td>
<td>6</td>
<td>3 Gy</td>
<td>2-3 h</td>
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<td>Syngeneic</td>
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<td>1.75</td>
<td>6</td>
<td>3 Gy</td>
<td>12-14 d</td>
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<td>6</td>
<td>3 Gy</td>
<td>12-14 d</td>
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<td>0.05</td>
<td>Syngeneic</td>
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</tr>
<tr>
<td>11</td>
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<td>1.75</td>
<td>3</td>
<td>3 Gy</td>
<td>2-3 h</td>
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<td>2-3 h</td>
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<td>2-3 h</td>
<td>Expanded</td>
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<td>Allogeneic</td>
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<td>3 Gy</td>
<td>12-14 d</td>
<td>Expanded</td>
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Figure 4. Suppression of the in vivo GVHD-like reaction with UC-MSCs. (A) For each experimental group listed in Table 2, the subscore values are shown as a heat map. The bars next to the heat map show the overall GVHD scores as the sum of all subscores. Group 16 was the most effective treatment group (arrow). (B) Detailed data for group 16 (black curves, open symbols), showing progressive changes in human CD45+, CD3+, and CD8+ events (% of leukocytes) (upper panel), as well as in clinical GVHD score, normalized weight, and EFS (lower panel). Each point represents one animal at a certain time point. The curves in the normalized weight plot are animal-specific. LOESS estimators (thick lines) and 95% confidence intervals (gray) are indicated. For comparison, data and LOESS estimators from the control groups that underwent transplantation only with (blue) or without 1.75 \( \times \) 10^6 UCB-MNCs (red) are shown. Weekly estimated FDR-adjusted P values from asymptotic Wilcoxon-Mann-Whitney tests (group 15 versus group 16) are indicated in the clinical GVHD score plot. *P < .05; **P < .01; ***P < .001. P_{EFS} = 0.00904, log-rank test between group 15 (GVHD control) and group 16.
suggesting that genetic or epigenetic identity between MSCs and target immune cells may affect the degree to which the GVHD-like reaction is suppressed. The mechanisms underlying this effect are as-yet unclear, but it will be interesting to characterize them further and to extend the studies to syngeneic HSC/MSC pairs from bone marrow.

The next important factor in the exploratory Cox PH model was the simultaneous transplantation of MSCs together with MNCs. The observed superiority of cotransplanted MSCs may be related to the environment into which they are introduced. It is possible that both the homing and efficacy of MSCs are detrimentally affected by preexisting tissue damage.

The effects of MSC dose and previous expansion were weak relative to identity and timing and were of borderline or no significance in the analysis reported here. Although this supports the activity of primary MSCs, it should be noted that the average yields of 2 x 10^5 to 3 x 10^5 colony-forming MSCs from a single UC are still insufficient to permit direct use in a clinical setting, in which these doses are typically used per kilogram of body weight [59]. For this reason, optimization of culture conditions to unveil or preserve maximum immunosuppressive activity is likely to be an important issue. In this context, it will be important in the future to identify the mechanisms responsible for the GVHD effect and to define the variables, including tissue source, donor-specific variations, and culture conditions, that can influence activity.

In summary, our present study demonstrates the activity of UC-MSCs to limit GVHD-like immune disease in an animal model, identifying UC-MSCs as a potential source of therapeutic MSCs. The effects of MSC dose and previous expansion were weak relative to identity and timing and were of borderline or no significance in the analysis reported here. Although this supports the activity of primary MSCs, it should be noted that the average yields of 2 x 10^5 to 3 x 10^5 colony-forming MSCs from a single UC are still insufficient to permit direct use in a clinical setting, in which these doses are typically used per kilogram of body weight [59]. For this reason, optimization of culture conditions to unveil or preserve maximum immunosuppressive activity is likely to be an important issue. In this context, it will be important in the future to identify the mechanisms responsible for the GVHD effect and to define the variables, including tissue source, donor-specific variations, and culture conditions, that can influence activity.

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Conflict of interest statement: A.H., M.H., and A.K. are employed by Vita 34 AG. Authorship statement: S.F., M.C., N.H., and U.S. conceived the study and designed the experiments. A.H., M.H., S.F., and N.H. designed and performed the experiments. A.H., M.H., S.F., and N.H. performed the experiments. M.H., L.S., M.C., A.H., N.H., and S.F. wrote the manuscript. M.H., L.S., M.C., N.H., A.H., U.S., and S.F. proofread and finalized the manuscript. M.H. and L.S. contributed equally to this work and should be considered co-first authors. M.C. and S.F. contributed equally to this work and share last authorship.

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