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G-CSF treatment of healthy pediatric donors affects their hematopoietic microenvironment through changes in bone marrow plasma cytokines and stromal cells

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ABSTRACT

Although G-CSF mobilized peripheral blood stem cell (PBSC) transplantation is commonly used in adults, bone marrow (BM) is still the preferred stem cell source in pediatric stem cell transplantation. Despite the fact that G-CSF is increasingly being used to enhance the hematopoietic stem/progenitor cell (HSPC) yield in BM transplantation (G-BM), the direct effects of G-CSF on the pediatric BM microenvironment have never been investigated. The BM hematopoietic niche provides the physical space where the HSPCs reside. This BM niche regulates HSPC quiescence and proliferation through direct interactions with other niche cells, including Mesenchymal Stromal Cells (MSCs). These cells have been shown to secrete a wide range of hematopoietic cytokines (CKs) and growth factors (GFs) involved in differentiation, retention and homing of hematopoietic cells. Here, we assessed changes in the BM microenvironment by measuring levels of 48 different CKs and GFs in G-BM and control BM (C-BM) plasma from pediatric donors. In addition, the effect of G-CSF on cell numbers and characteristics of HSPCs and MSCs was assessed. IL-16, SCGF-b, MIP-1b (all >1000 pg/mL) and RANTES (>10.000 pg/mL) were highly expressed in healthy donor pediatric BM plasma. Levels of IL-3, IL-18, GROa, MCP-3 ($p<0.05$) were increased in G-BM, whereas levels of RANTES ($p<0.001$) decreased after G-CSF treatment. We found a negative correlation with increasing age for IL2-Ra and LIF ($p<0.05$). In addition, a concomitant increase in the number of both hematopoietic and fibroblast colony forming units was observed, indicating that G-CSF affects both HSPC and MSC numbers. In conclusion, G-CSF treatment of healthy pediatric donors affects the hematopoietic BM microenvironment by expansion of HSPC and MSC numbers and modifying local CK and GF levels.

1. Introduction

The bone marrow (BM) microenvironment or niche regulates hematopoietic stem and progenitor cell (HSPC) homeostasis under physiologic conditions and coordinates their behavior in response to stress. The hematopoietic niche contains different stromal populations including endothelial cells (ECs), mesenchymal stromal cells (MSCs), and osteoblastic cells (OBs), which produce pivotal HSPC-supporting factors such as attachment factors, extracellular matrix components, hematopoietic growth factors (GFs), and cytokines (CKs) [1–6].

In contrast to the G-CSF mobilized peripheral blood stem cell (PBSC) transplantations that are commonly used in adults [7], BM remains the leading stem cell source for pediatric transplantation [8]. Although G-CSF is increasingly being used to enhance the HSPC yield of BM, there are few data on the direct effects of G-CSF on the pediatric hematopoietic niche. Several studies have addressed the beneficial roles of G-CSF-primed bone marrow (G-BM), and have demonstrated accelerated engraftment, lower incidence of acute/chronic graft versus host disease (GvHD), and improved survival [9–12]. G-CSF stimulation induces a cascade of events, starting with the expansion of neutrophils and their

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precursors, followed by controlled release of proteolytic enzymes, which cleave and deactivate many of the attachment proteins that anchor HSPCs to their niche [13–15]. G-CSF-induced suppression of OBs and activation of osteoclasts (OCs) and CD169+ BM macrophages results in the production of Stromal Derived Factor-1 (SDF-1) by Nestin+ MSCs and causes mobilization of the HSCPs to peripheral circulation [7,13,16–18].

MSCs are key regulators of the hematopoietic microenvironment [19]. They are multipotent cells with the capacity to differentiate into OBs, adipocytes, and chondrocytes [20], but also function as hematopoietic supporting cells [21]. MSCs display a high potential for self-renewal [22], as reflected by their *in vitro* colony-forming-unit fibroblast (CFU-F) activity [23]. MSCs regulate homing of HSPCs through secretion of SDF-1 and Stem Cell Factor (SCF) [24,25] and support maintenance of HSPCs via production of extracellular matrix molecules (ECM) and hematopoietic GFs [1].

In order to better understand how HSPCs are regulated in their microenvironment and to improve the efficacy of (future) stem cell therapies, it is crucial to identify the factors that affect HSPCs and modulate maintenance, quiescence, proliferation, differentiation, migration, and homing. Although the effects of G-CSF on mobilization of HPSCs are well known, there is little detailed information about the direct effects of G-CSF on the pediatric bone marrow microenvironment.

Here, we assessed plasma from G-CSF-primed (G-BM) and unstimulated control BM (C-BM) from healthy pediatric donors for differences in levels of CKs and GFs. We also monitored differences in HSPC and MSC numbers and differentiation capacity. These data may contribute to a better understanding of the effects of G-CSF on the pediatric hematopoietic BM niche.

2. Materials and methods

2.1. Subjects and ethical approval

Bone marrow samples (2–5 mL) were collected from healthy G-CSF-treated (n=13) and untreated pediatric donors (n=40), scheduled for transplantation-related harvest. When the predicted harvest volume was expected to exceed 20 mL/kg of donor weight, 10 μg/kg for 3 days G-CSF (Filgastrim, Neupogen) was administered. Ethical approval was obtained from the Hacettepe University Institutional Ethics Committee (HEK12/192–04) and informed consent forms were signed for each sample. All procedures were done in accordance with the Helsinki Declaration.

2.2. Bone marrow plasma

BM samples were centrifuged at 1500 rpm, for 10 min at room temperature (Eppendorf Centrifuge 5810R, Hamburg, Germany) to collect plasma. Cell-free samples were aliquoted and stored at −80 °C.

2.3. Colony forming unit assays

BM mononuclear cells (BM-MNC) were isolated with Biocoll (1.077 g/L; Biochrom AG, Berlin, Germany). Hematopoietic colony assays were performed in non-tissue culture treated 35 mm Petri dishes (# BD351008) using serum-free semi-solid methylcellulose cultures (MethoCult™ H4434 Classic, Stem Cell Technologies, Vancouver, Canada), that support myeloid and erythroid differentiation of HSPCs. In contrast, colony forming unit-fibroblast (CFU-F) assays were performed in tissue culture treated 6-well plates (Corning, Costar #3516) using DMF10, consisting of 60% DMEM-LG (Gibco, Paisley, UK)/40% MCDB-201 (Sigma, MO, USA), 10% heat-inactivated fetal bovine serum (FBS-HI; Gibco, UK), 2 mM L-Glutamine (Irvine Scientific, USA) and 1% penicillin/streptomycin (Biochrom AG, Germany). Colonies were counted between days 10–14.

2.4. Cell culture

MSCs were isolated from G-BM and C-BM and maintained in DMF10. Upon subconfluence, cells were harvested with 0.25% Trypsin/1 mM EDTA and replated at a concentration of $1.3x10^3$ cells/cm^2. All analyses were performed with passage 3 (P3) cells. Population doublings were calculated at the end of each passage using the following equation: n = log (AC-BC)/log2 (AC: cell count after culture; BC: cell count before culture). This method is commonly used for determination of population doublings in MSCs [26].

2.5. Differentiation assays

For adipogenic and osteogenic differentiation, cells were maintained for 21 days in differentiation media, as previously described [27]. Images were obtained with an inverted microscope (Olympus CKX41; Olympus, Tokyo, Japan). Oil Red O (ORO) content was measured spectrophotometrically at 492 nm with a Sunrise™ Microplate Reader (Tecan Groups Ltd, Männedorf, Switzerland). Calcium content was assessed using the Quantichrom™ Calcium Assay kit (DICA500, BioAssay Systems, Hayward, CA, USA) and measured at 620 nm. Data were analyzed with Magellan™ data analysis software.

2.6. Flow cytometry

Flow cytometric analysis was performed using MSCs derived from age-matched G-BM (n = 13) and C-BM (n = 13) using a FACSARIA (Becton Dickinson, NJ, USA). Single cells were incubated in phosphate-buffered saline (PBS), 0.05% NaN_3, 0.5% Bovine Serum Albumin and 2% human AB serum in presence of CD29, CD44, CD90, CD106, CD144, CD146, CD166 from Biolegend (San Diego, CA, US), CD14, CD31, CD34, CD45, CD73, CD140b, CD200, HLA-DR and CD271 from BD Biosciences (San Diego, CA, US) and CD105 and CD133-PE from e-Bioscience (San Diego, CA, US). Events were analyzed using FACSDIVA software (Becton Dickinson, NJ, US).

2.7. Multiplex and ELISA assays

To determine plasma cytokine levels in age-matched G-BM (n = 10, age 9.5 ± 4.8 years) and C-BM (n = 10, age 10.4 ± 3.5 years), the Bio-Plex Pro Human Cytokine 27-plex Assay (M50-0KCAF0Y, Bio-Rad) was used to measure bFGF, Eotaxin, G-CSF, GM-CSF, IFNγ, IL-1β, IL-1Rα, IL-2, IL-4, IL-5, Il-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, IP-10, MCP-1(MCAF), MIP-1α, MIP-1β, PDGF-BB, RANTES, TNFα, VEGF and the Bio-Plex Pro Human Cytokine 21-plex Assay (MF0-005KMII, Bio-Rad) was used to measure IL-1α, IL-2Rα, IL-3, IL-12(p40), IL-16, IL-18, CTACK, GROα, HGF, IFNα2, LIF, MCP-3, M−CSF, MIF, MIG, βNGF, SCF, SCGFβ, SDF-1α, TNFβ, TRAIL. Plates were read using xMAP technology on a Bio-Plex MAGPIX multiplate reader and analyzed using Bio-Plex Manager™ Software and Bio-Plex Data Pro™ Software (Bio-Rad, CA, USA). Separate ELISA's were performed for APO-1/FAS (#KHS9502/KHS9501, BioSource Int. Wilmington, NC, US), SDF-1α (Quantikine® kit DSA00, R&D systems, Minneapolis, MN, US) and IL-7 (#KHC0071, Invitrogen, Carlsbad, CA, US), according to the manufacturer's instructions.

2.8. Statistics

Statistical analyses were performed using Microsoft Excel for Mac 2011 or SPSS 6.1. Correlations were calculated using Spearman's correlation. P-values for comparisons between two groups were calculated using an unpaired T-test. Multiplex data were analyzed with the Kruskal-Wallis one-way analysis of variance, using Bio-Plex Data Pro™ Software (Bio-Rad, CA, US).

3. Results

3.1. G-CSF treatment increases IL-3, IL-18, GROa, and MCP3 and decreases RANTES levels in healthy pediatric BM plasma

To assess the effects of G-CSF on CK and GF levels in BM plasma, a multiplex-cytokine array was performed using healthy pediatric G-BM plasma and age-matched C-BM plasma (Table 1). IL-16, SCGF-b, MIP-1b (all >1000 pg/mL), and RANTES (>10.000 pg/mL) were strongly expressed in C-BM plasma. A negative correlation with increasing age was found for levels of IL2-Ra (p=0.045) and LIF (p= 0.032), as shown in Fig. 1. After G-CSF treatment, levels of G-CSF in BM plasma increased approximately three-fold (p<0.05). In addition, levels of IL-3, IL-18, GROa, and MCP-3 significantly increased in G-BM (p<0.05), whereas RANTES levels in G-BM plasma decreased significantly (p< 0.001). Other CKs and GFs were not significantly affected by G-CSF treatment, but are given in Table 1. Differences in BM plasma cytokine levels from superficial (BM-1) and deep (BM-2) punctures in untreated C-BM donors were also assessed. C-BM plasma levels of IFN-γ, IL-3, IL-4, IL-5, IL-8, IL-12(p40), PDGF-BB, TNFα, and RANTES were all significantly lower (p<0.05) in BM-1 samples, whereas levels of HGF and b-NGF were significantly higher in BM-1 samples (p<0.05) compared to BM-2 samples (Supplemental Table 1). Levels of IL-7 (p<0.001), SDF1α (p<0.0001) and soluble APO1-FAS (p<0.02) were consistently higher in the BM-1 plasma samples (Supplemental Fig. 1). Thus, in comparison to age-matched C-BM plasma, levels of the pro-inflammatory cytokines IL-3, IL-18, GROa, and MCP3 in G-BM were significantly higher, whereas levels of the chemokine RANTES were significantly lower.

3.2. G-CSF treatment increases BM CFU numbers of both HSPCs and MSCs

We performed colony assays to quantify the effect of G-CSF on numbers of HSPCs and MSCs and analyze their relationship. Quantities of CFU-GM (p<0.01), BFU-E (p<0.01) and CFU-F (p<0.0001) were significantly higher in G-BM than in C-BM samples (Fig. 2). The correlations (r) between CFU-F and CFU-GM numbers and CFU-F and BFU-E numbers were 0.62 (p<0.02) and 0.63 (p<0.01), respectively. These data indicate that G-CSF treatment results in a simultaneous increase in both BM-HSPCs and BM-MSCs. The effects of G-CSF treatment on the BM hematopoietic niche are summarized in Fig. 3.

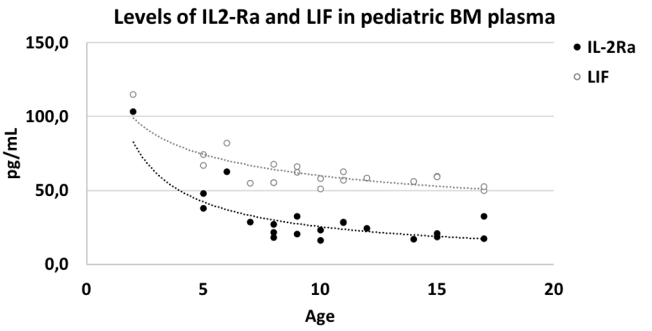

Fig. 1. Effect of age on BM plasma levels of IL2-Ra and LIF in healthy children. Pediatric BM plasma levels of IL2-Ra (black, filled circles) and LIF (grey, open circles) were negatively correlated with age (p<0.05).

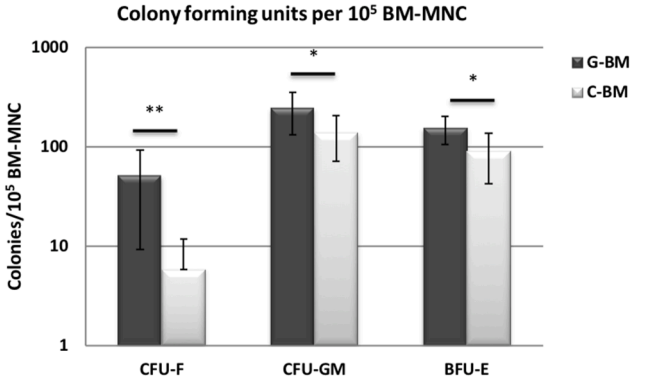

Fig. 2. Colony forming units in G-CSF treated pediatric BM donors and controls. Number of colony forming units (CFU) per 10^5 BM mononuclear cells (MNC) on the y-axis. CFU-F (Colony Forming Unit-Fibroblast), CFU-GM (Colony Forming Unit-Granulocyte/ Macrophage) and BFU-E (Burst Forming Unit-Erythroid) in G-CSF treated BM donors (G-BM, n=6, dark grey) and untreated controls (C-BM, n=24, light grey). Data are given as mean ± standard deviation. * p<0.01; ** p<0.0001.

Table 1
Cytokine levels (pg/mL) in C-BM and G-BM plasma.

Cytokines (pg/mL)	C-BM plasma	G-BM plasma	p-values	Cytokines (pg/mL)	C-BM plasma	G-BM plasma	p-values
IL-1α	42.3 ± 19.7	45.3 ± 18.7	0.73	**GM-CSF**	95.6 ± 35.0	110.3 ± 32.4	0.34
IL-1β	41.1 ± 14.6	243.3 ± 303.9	0.05	**GROa**	53.6 ± 8.5	62.6 ± 9.3	0.036
IL-1Rα	73.3 ± 56.6	69.8 ± 36.5	0.87	**HGF**	758.8 ± 333.4	1060.5 ± 396.0	0.08
IL-2	104.9 ± 64.1	95.1 ± 41.8	0.69	**IFNα2**	44.39 ± 12.85	46.40 ± 20.91	0.45
IL-2Rα	26.2 ± 8.8	36.3 ± 27.3	0.28	**IFNγ**	24.0 ± 5.2	22.5 ± 2.4	0.43
IL-3	42.1 ± 3.5	49.5 ± 9.8	0.037	**IP-10**	331.1 ± 336.3	189.1 ± 123.2	0.23
IL-4	29.2 ± 4.3	29.6 ± 3.4	0.83	**LIF**	58.0 ± 5.5	68.2 ± 18.8	0.12
IL-5	12.6 ± 3.3	12.3 ± 1.6	0.76	**MCP-1**	474.1 ± 178.0	488.6 ± 171.0	0.85
IL-6	84.8 ± 76.1	138.0 ± 131.0	0.28	**MCP-3**	22.2 ± 2.5	26.6 ± 3.1	0.003
IL-7	32.0 ± 46.0	68.1 ± 154.2	0.49	**M−CSF**	80.9 ± 20.1	105.6 ± 37.7	0.08
IL-8	26.7 ± 6.0	41.8 ± 32.5	0.17	**MIF**	701.6 ± 965.8	398.9 ± 140.8	0.34
IL-9	142.7 ± 63.5	133.2 ± 22.8	0.66	**MIG**	569.2 ± 596.2	441.1 ± 431.5	0.59
IL-10	129.4 ± 221.0	185.2 ± 400.2	0.70	**MIP-1α**	28.8 ± 7.7	26.1 ± 2.4	0.30
IL-12p40	66.5 ± 19.0	78.1 ± 16.6	0.16	**MIP-1b**	1151.5 ± 521.1	1617.0 ± 543.8	0.07
IL-12p70	103.7 ± 86.7	204.1 ± 423.1	0.47	**bNGF**	25.5 ± 5.6	27.2 ± 6.4	0.53
IL-13	59.0 ± 49.7	123.8 ± 178.1	0.28	**PDGFbb**	857.5 ± 498.0	716.7 ± 197.4	0.42
IL-15	126.4 ± 51.5	118.1 ± 30.4	0.66	**RANTES**	12270.0 ± 1256.1	9838.2 ± 1377.9	0.0006
IL-16	1018.3 ± 417.5	778.8 ± 254.6	0.14	**SCF**	178.2 ± 46.5	173.4 ± 61.8	0.85
IL-17	89.4 ± 10.6	88.7 ± 8.3	0.89	**SCGFb**	2979.5 ± 1106.0	2844.4 ± 1040.3	0.78
IL-18	290.0 ± 93.8	1710.0 ± 2018.8	0.039	**SDF1α**	14.4 ± 2.6	17.4 ± 4.1	0.07
CTACK	130.3 ± 86.9	82.8 ± 25.4	0.11	**TNFα**	25.7 ± 2.9	25.0 ± 2.3	0.55
Eotaxin	82.6 ± 42.8	61.9 ± 14.0	0.16	**TNFβ**	28.2 ± 6.4	33.3 ± 10.1	0.19
bFGF	158.1 ± 61.0	157.4 ± 88.1	0.98	**TRAIL**	63.2 ± 18.2	72.0 ± 22.1	0.34
G-CSF	647.7 ± 1173.0	2051.0 ± 1512.7	0.032	**VEGF**	260.9 ± 111.5	267.8 ± 79.8	0.88

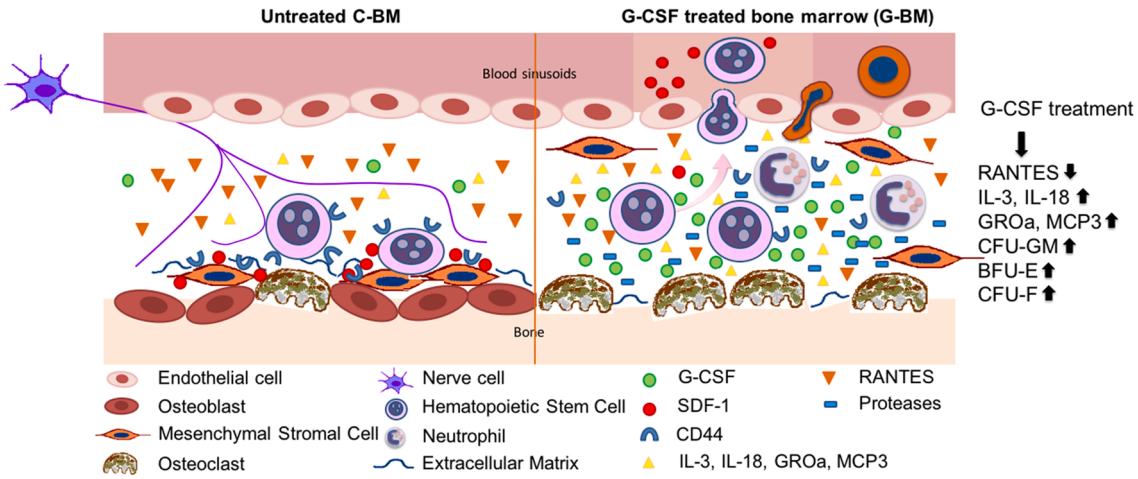

Fig. 3. The effects of G-CSF treatment on the healthy, pediatric bone marrow niche. Left: Physiological situation in untreated, control bone marrow (C-BM); Right: BM niche after G-CSF treatment (G-BM). G-CSF treatment of healthy pediatric donors results in a significant increase in the levels of IL-3, IL-18, GROa, and MCP3 in BM plasma ($p<0.05$), a significant decrease in RANTES ($p<0.001$), and a concomitant increase in both hematopoietic and mesenchymal colony forming units (CFU) with subsequent release of both HSPCs and MSCs to the peripheral blood.

3.3. G-CSF does not affect immunophenotype or differentiation capacity of BM-MSCs

To assess whether G-CSF has long-term effects on the stromal elements of the hematopoietic microenvironment, MSCs from G-BM (n=13) and C-BM (n=18) were immunophenotyped at the end of passage 3. MSCs from both groups showed high expression (>92%) of typical MSC markers such as CD29, CD44, CD73, CD90, CD105, and CD166 (Table 2, Supplemental Fig. 2) and lacked expressions of hematopoietic or endothelial cell markers. Significant differences in the expression of surface antigens by MSCs from the G-CSF treated and control group were not detected. Thus, G-CSF treatment did not appear to have any long-term effects on the immunophenotype of MSCs. Similarly, G-CSF did not affect MSC differentiation potential, since G-BM MSCs displayed an adipogenic (0.5 ± 0.2 vs 0.5 ± 0.3 mg/mL ORO for G-BM and C-BM, respectively) and osteogenic (15.7 ± 7.4 vs 13.2 ± 6.2 calcium mg/dL for G-BM and C-BM, respectively) differentiation capacity comparable to C-BM MSCs.

Table 2
Phenotype (%) of BM-MSCs obtained from healthy pediatric control (C-BM) and G-CSF treated (G-BM) bone marrow donors. MSCs: Mesenchymal stromal cells, HSPCs: hematopoietic stem/progenitor cells, HSCs: Hematopoietic stem cells, ESCs: Embryonic stem cells.

Antigen (alternative name)	Expressed by	C-BM (n=18) %	G-BM (n=13) %
CD29 (Integrin β1)	MSCs	94.1 ± 6.2	94.9 ± 4.7
CD31 (PECAM-1)	Endothelial cells	0.1 ± 0.2	0.3 ± 0.3
CD34	HSPCs, endothelial cells	0.2 ± 0.3	0.2 ± 0.4
CD44 (HCAM)	MSCs, T cells	92.5 ± 5.8	93.8 ± 3.7
CD45	Hematopoietic cells	0.2 ± 0.3	0.4 ± 0.4
CD73 (5′-nucleotidase)	MSCs, T cells	93.9 ± 7.1	98.1 ± 1.7
CD90 (Thy-1)	HSCs, MSCs	98.1 ± 2.1	98.6 ± 1.8
CD105 (Endoglin)	MSCs, endothelial cells	95.8 ± 6.6	96.7 ± 3.3
CD106 (VCAM-1)	MSCs, smooth muscle cells, vascular endothelium	2.5 ± 2.5	3.6 ± 4.5
CD133 (Prominin-1)	HSCs, MSCs	0.1 ± 0.2	0.3 ± 0.3
CD140b (PDGFR-B)	MSCs, fibroblasts	76.6 ± 22.5	79.9 ± 21.6
CD144 (VE-Cadherin)	Endothelial cells	0.2 ± 0.3	0.5 ± 0.7
CD146 (MCAM)	MSCs	41.2 ± 28.5	50.7 ± 28.8
CD166 (ALCAM)	MSCs, T cells	94.5 ± 6.1	95.5 ± 2.6
CD200 (OX-2)	B cells, T cells, neuronal cells, endothelial cells	8.9 ± 8.1	11.4 ± 8.1
CD271 (LNGFR)	MSCs, hematopoietic cells	2.1 ± 2.4	6.8 ± 14.0
ALP (Alkaline Phosphatase)	ESCs, osteogenic cells	5.2 ± 10.3	11.1 ± 20.4
HLA-ABC	All nucleated cells, platelets	94.9 ± 8.4	96.9 ± 1.8
HLA-DR	Antigen presenting cells	3.7 ± 9.2	0.4 ± 0.4

3.4. BM-MSC from G-CSF treated donors display decreased cumulative population doublings

Since the data suggested *in vivo* expansion of the number of multipotent stromal progenitor cells in G-BM, we wanted to assess the proliferative capacity of these cells in long-term *in vitro* cultures. Population doublings of BM-MSCs from G-BM and C-BM samples were measured from passage 1 to passage 10 (Fig. 4). Although population doublings were similar up to passage 5, G-BM MSCs displayed less frequent population doublings from passage 6 onwards in comparison to C-BM MSCs ($p<0.02$). Furthermore, G-BM and C-BM MSCs reached a cumulative population doubling number of 20.3 and 25.1, respectively. Thus, although G-CSF treatment does not affect MSC immunophenotype or differentiation, it may have an effect on the proliferative capacity of

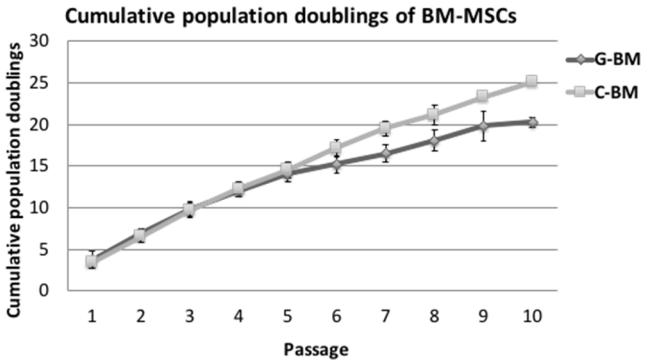

Fig. 4. Cumulative population doublings of healthy pediatric G-BM and C-BM MSCs. Population doublings were calculated for up to ten passages using the following formula: n = log (AC-BC)/log2 (AC: cell count after culture, BC: cell count before culture). Plating density was kept constant at 1333 cells/cm^2. Population doublings were calculated for cells during the log phase of expansion. Data shown are from untreated healthy pediatric BM donor (C-BM, light grey, n=22) and G-CSF treated donor (G-BM, dark grey, n=8) BM-MSCs. Data are given as mean ± standard deviation.

these cells.

4. Discussion

G-CSF-primed BM (G-BM) has been used effectively as an alternative to G-CSF-mobilized PB cells in adult donors. It has also been suggested for pediatric donors because of its advantages with respect to ease of collection for the donor (no need for central line placement), accelerated engraftment, and decreased risk of fatal GvHD in the recipient [28–30]. Two prospective, multicenter studies using G-BM from pediatric donors as the primary cell source for HSC transplantation demonstrated the safety and feasibility of this procedure, which facilitated engraftment and lowered GvHD risk [10,11]. However, until now, the effects of G-CSF treatment on the BM hematopoietic microenvironment of healthy pediatric donors have not been assessed directly. Under standard conditions, there may be no need to collect G-CSF-primed marrow for HSC transplantation. However, in cases when donor weight is low, the volume of the harvested marrow may not contain sufficient cells for engraftment, since the maximum volume of harvest should not exceed 20 mL/kg. In these donors, G-CSF can be used pre-harvest to increase the number of HSPCs in the primed BM product. Assessing the effects of G-CSF on BM of healthy pediatric donors may contribute to a better understanding of the hematopoietic microenvironment and help optimize BM harvest protocols. Other indications for use of G-CSF include alleviating neutropenia in severe congenital neutropenia (SCN) syndromes [31]. Treatment of these patients with G-CSF has been shown to alleviate SCN symptoms, reduce the infection rate, and increase quality of life in 90% of patients. However, despite G-CSF treatment, patients retain an increased risk of sepsis, which is thought to be related to the functional defects of neutrophils.

In this paper, we evaluated the effects of G-CSF on GF and CK levels in BM plasma of healthy pediatric donors. We also assessed the numbers of HSPCs and MSCs in BM and the long-term effects of G-CSF treatment on BM-MSCs to gain a better understanding of the effects of G-CSF treatment on the healthy BM hematopoietic environment. We found high expression of IL-16, SCGF-b, MIP-1b, and RANTES in healthy pediatric donor BM samples, a negative correlation with increasing age for levels of IL2-Ra and LIF, and a significant increase in the levels of the pro-inflammatory cytokines IL-3, IL-18, GROa, and MCP3 after G-CSF priming, which coincided with a significant decrease in the levels of the chemokine RANTES. In addition, G-CSF treatment resulted in a concomitant increase in numbers of CFU-GM, BFU-E, and CFU-F, indicating a stimulatory effect on both HSPCs and MSCs. This is in line with data showing that treating mice and healthy donors with G-CSF results in the simultaneous expansion of both hematopoietic and mesenchymal progenitors in the BM and their mobilization to the PB [32–35].

G-CSF has been proven to be very effective for mobilizing HSPCs. The effects of G-CSF on cell populations and CK levels in the peripheral blood are well documented [34,36,37]. G-CSF treatment of healthy donors results in a significant increase of interleukin-6 (IL-6), IL-8, and IL-10 and a decrease in Tumor Necrosis Factor-alpha (TNFα), IFN-γ, and GM-CSF in PB. In addition, plasma levels of TNFα, soluble TNF receptors (sTNF-R) p55 and p75, and IL-IRα increase significantly [36,37]. Measurements of supernatants from primary BM and PB cultures of G-CSF-treated animals showed also a significant difference in cytokine expression profiles with increased TNFα and IL-17 levels in BM and enhanced levels of IL-10 and IFNγ in PB cultures, indicating that G-CSF stimulated cells derived from BM and PB may have functionally different properties [34].

Our data further show that G-CSF induced the production of the pro-inflammatory cytokines IL-3 and IL-18 in BM plasma. This suggests that G-CSF may, depending on the situation, play a dual role in modulating inflammation, and that the effects of G-CSF on BM and PB may be divergent. Interestingly, we also found a significant increase in HGF and MIF levels after G-CSF treatment, but only in plasma samples obtained from superficial BM punctures. These data support the idea that G-CSF may have differential effects on distinct hematopoietic niches, depending on the types of cells that dominate the niche.

RANTES, commonly known as a chemotactic cytokine, is predominantly produced by BM stromal cells, osteoblasts, and platelets. Whereas its chemotactic functions are mediated at low concentrations, at higher levels RANTES appears to function as a mitogen [38]. Patients with severe BM failure syndromes display higher levels of serum G-CSF and Flt3-ligand and lower RANTES levels compared to healthy controls [39]. RANTES-deficient mice displayed severe osteopenia [40] and a strong increase in lymphoid-biased HSCs [41]. In the present study, we found that G-CSF priming resulted in significantly decreased levels of RANTES, which may be related to the direct effects of G-CSF on OBs [15,17] and the secretory profile of MSCs [42].

G-CSF has been shown to modulate MSC behavior and activation through immediate interaction with the G-CSF-receptor on the MSC surface [14]. Although we and others have found that treatment with G-CSF did not affect BM-MSC morphology, immunophenotype, or differentiation capacity [42], we did find an effect on CFU-F numbers and MSC proliferation. Since this was not apparent in early passages, it may have been missed by other studies [33]. G-BM MSCs showed a significantly decreased cumulative population doubling potential, indicating that the early stimulating effects of G-CSF on MSCs may increase initial CFU-F numbers in BM, but may subsequently result in exhaustive expansion and early senescence. Since MSCs from both groups were age-matched and cultured under the same conditions, the G-CSF treatment is the most likely reason for the differences between G-BM and C-BM MSCs. G-CSF has been shown to affect MSC proliferation in *in vitro* animal models [43,44] and therefore exhaustion, resulting in preliminary senescence, appears to be the most likely explanation. Mobilization of BM-MSCs using G-CSF is being investigated for its potential to treat of a wide range of diseases including brain injury [45,46] and heart disease [32,47]. These protocols do not require *ex vivo* expansion of BM-MSCs and therefore exhaustion may not play a major role in clinical use. However, the use of G-CSF to mobilize or stimulate BM-MSCs, followed by expansion cultures and subsequent use for immune-modulating purposes, may need to be further explored.

In conclusion, G-CSF treatment of healthy donors results in a simultaneous and correlated increase in both HSPCs and MSCs and promotes a pro-inflammatory, secretory profile within the hematopoietic BM niche.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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