Mesenchymal stromal cells induce a permissive state in the bone marrow that enhances G-CSF-induced hematopoietic stem cell mobilization in mice

Running title: MSC induce a permissive state in the bone marrow

Evert-Jan F.M. de Kruijf, Rob Zuijderduijn, Marjolein C. Stip, Willem E. Fibbe and Melissa van Pel
Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands

Author contributions:
Evert-Jan F.M. de Kruijf: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of the manuscript
Rob Zuijderduijn: collection and/or assembly of data, final approval of manuscript
Marjolein C. Stip: collection and/or assembly of data, final approval of manuscript
Willem E. Fibbe: conception and design, final approval of the manuscript
Melissa van Pel: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of the manuscript

Corresponding author:
M. van Pel, Ph.D.
Department of Immunohematology and Blood transfusion
Leiden University Medical Center
PO Box 9600, 2300 RC Leiden
The Netherlands
Tel: (+31) 71-5265277 / Fax: (+31) 71-5265267
Email: m.van_pel@lumc.nl

Abstract

Mesenchymal stromal cells (MSC) support hematopoietic stem cells (HSC) *in vivo* and enhance HSC engraftment and hematopoietic recovery upon co-transplantation with HSC. These data have led to the hypothesis that MSC may impact the HSC niche, leading to changes in HSC retention and trafficking. We studied the effect of MSC administration on the HSC compartment in the bone marrow (BM) in mice. Following injection of MSC, HSC numbers in the BM were decreased coinciding with an increased cell cycle activity compared to PBS-injected controls. Furthermore, the frequency of macrophages was significantly reduced and niche factors including *Cxcl12*, *Scf* and *Vcam* were downregulated in endosteal cells. These BM changes are reminiscent to events associated with G-CSF-induced hematopoietic stem –and progenitor cell (HSPC) mobilization. Interestingly, co-administration of MSC and G-CSF resulted in a two-fold increase in peripheral blood HSPC release compared to injection of G-CSF alone, whereas injection of MSC alone did not induce HSPC mobilization. After intravenous administration, MSC were only observed in the lungs, suggesting that MSC exert their effect on the HSC niche through a soluble mediator. Therefore, we tested the hypothesis that MSC-derived extracellular vesicles (EV) would be responsible for the observed changes in the HSC niche. Indeed, administration of EV resulted in downregulation of *Cxcl12*, *Scf* and *Vcam* and enhanced G-CSF-induced HSPC mobilization at similar levels as compared to MSC and G-CSF. Together, these data indicate that MSC induce a permissive state in the BM enhancing HSPC mobilization through the release of EV.

Introduction

Hematopoietic stem cells (HSC) replenish the peripheral blood (PB) cell pool throughout life. During homeostasis, the vast majority of HSC reside in specialized niches, located in the perivascular area of the trabeculated region of the bone marrow (BM). This HSC microenvironment regulates self-renewal, cell cycle entry and differentiation of HSC and consists of a complex network of hematopoietic and non-hematopoietic cells. (reviewed in [1,2]).

In the BM, the majority of HSC are found in close proximity to MSC surrounding arterioles and sinusoids [3-6]. MSC-derived CXCL12 and Stem Cell Factor (SCF) are indispensable for HSC maintenance as deletion of either CXCL12 or SCF leads to hematopoietic exhaustion [7-11]. HSC are retained in the niche by adhesion molecules, including β1-integrins, interacting with extracellular matrix components and with VCAM, expressed on stromal cells [12].

The endosteal region of the BM contains a population of resident macrophages (osteal macrophages or osteomacs) supporting osteoblast differentiation and mineralization and contributing to the maintenance of HSC niches [13]. Another BM-resident macrophage population, expressing CD169, supports the retention of HSC by acting on stromal cells in the niche [14]. Depletion of osteomacs or $CD169^{+}$ macrophages results in downregulation of *Cxcl12*, *Vcam, Ang-1* and *Scf* and results in subsequent hematopoietic stem -and progenitor cell (HSPC) mobilization [13-15].

Through administration of exogenous cytokines, HSPC can be induced to leave the niche and migrate towards the PB, a process called mobilization. Granulocyte-colony stimulating factor (G-CSF) is most commonly applied as a mobilizing agent.

Administration of G-CSF is accompanied by neutrophil expansion and a proteolytic BM milieu, coinciding with decreased levels of the protease inhibitor alpha-1-antitrypsin (AAT)

[16,17]. Simultaneously with neutrophil expansion, G-CSF administration leads to depletion of macrophages resulting in decreased expression of *Cxcl12*, *Vcam* and *Scf* by BM stromal cells and in decreased osteoblast numbers [14,15]. Together, these events result in decreased adhesion of HSPC to their niche and as a consequence HSPC migrate towards the PB.

MSC are a non-hematopoietic population of cells that form CFU-F and have the capacity to differentiate into osteoblasts, adipocytes and chondrocytes. MSC can be isolated from the BM, where they are an essential part of the hematopoietic stem cell niche [2]. When co-transplanted with $CD34^+$ umbilical cord blood-derived HSPC, MSC enhance both HSC engraftment and hematopoietic recovery [18,19]. While the underlying mechanisms are not fully understood, it was suggested that HSC homeostasis is indirectly altered through factors released by the injected MSC, since intravenously injected MSC could not be detected in the BM after administration [19].

Given the key role of MSC in the HSC microenvironment and their effect on HSC engraftment and hematopoietic recovery, we have investigated the effect of MSC administration on the hematopoietic BM compartment. Here we show that intravenous administration of MSC results in changes in the BM that are reminiscent to events that occur during G-CSF-induced HSPC mobilization. Furthermore, co-injection of MSC and G-CSF synergistically enhanced HSPC mobilization, compared to G-CSF alone. MSC retained in the lung exerted their effects on the BM through the secretion of extracellular vesicles (EV). Administration of EV alone resulted in downregulation of *Cxcl12*, *Scf* and *Vcam* and enhanced G-CSF-induced HSPC mobilization at similar levels as compared to MSC. Together, these data indicate that MSC administration induces a permissive state in the BM through the release of EV, promoting HSPC mobilization.

Materials and methods

Animals

Eight to 12-week-old male C57BL/6-Ly5.2 and C57BL/6-Ly5.1 mice were obtained from Charles River Laboratories (Maastricht, The Netherlands). The animals were fed commercial rodent chow and acidified water ad libitum and were maintained in the animal facility of the LUMC under conventional conditions. All experimental protocols were approved by the institutional ethics committee on animal experiments.

Mesenchymal stromal cells

MSC were obtained by culturing bone chips in a 75 cm^2 flask in MSC medium, containing αMEM (Life Technologies), 10% FCS, penicillin/streptomycin and L-Glutamine. Plastic adherent MSC were cultured to 95% confluency in a fully humidified atmosphere at 37°C and 5% CO_2, harvested using trypsin and further expanded until sufficient numbers were obtained. MSC used throughout this study were of passage six to ten. MSC were administered intravenously in 0.1% Bovine Serum Albumin/Phosphate Buffered Saline (0.1% BSA/PBS) at a dose of 200 $x10^3$ cells per day for three consecutive days. Mice injected with 0.1% BSA/PBS served as controls. In indicated experiments, MSC were cultured in the presence of recombinant murine IFN-γ (20 ng/ml) or recombinant murine TNF-α (20 ng/ml; both R&D Systems, Abingdon, UK) for 7 days. Where indicated, MSC were transduced with a lentiviral vector containing *SFFV-DsRed-Firefly luciferase (SFFV-DsR-Fluc)* as previously described [20]. Images were acquired and analyzed as previously described [20]. To obtain MSC culture supernatant, MSC at a confluency of 70-80% were cultured for 1 week in StemSpan (Stemcell Technologies, Köln, Germany). Subsequently, the medium was harvested, centrifuged to deplete for cell debris and concentrated using Centriprep YM3 filters

(Millipore, Amsterdam, the Netherlands) to obtain a ~20-fold concentration. In indicated experiments, 200 µl of MSC culture supernatant was administered intraperitoneally twice daily for 3 consecutive days.

Cell lines

RAW264.7 cells (gift from A. van Wengen, LUMC) were cultured in RPMI-1640 containing 10% FCS, penicillin, streptomycin and L-glutamine. S17 and MS-5 cells (gift from F.J.T. Staal, LUMC) were cultured in MSC medium and MSC medium with 50 µM 2-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, The Netherlands) respectively.

In co-culture experiments, 35 $x10^3$ stromal cells were cultured in their respective medium for 16h. Medium was removed, RAW264.7 cells were added in a 1:1 ratio and cultured for 72 hours in MSC medium. RAW264.7 cells were either directly added to the stromal cells or cultured in transwells with a 0.4 µm pore size (Corning Costar). Stromal cells were harvested using Accumax (eBioscience). RAW264.7 cells were depleted using CD45-microbeads (Miltenyi, Leiden, The Netherlands) and MACS separation.

Preparation of cell suspensions and BM extracellular extracts

Twenty-two to 24 hours after the last MSC administration, mice were sacrificed by CO_2 asphyxiation. PB was obtained by intracardiac puncture and cell counts were performed on a Sysmex XP-300 counter (Sysmex, Etten-Leur, The Netherlands). PB was centrifuged at 350xg and blood plasma was stored at -20 ^oC. Erythrocytes were lysed using a specific lysis buffer (LUMC Pharmacy, Leiden, The Netherlands) prior to further analysis.

BM and spleen cells were harvested as previously described [21].

BM extracellular extracts were obtained by flushing femurs with 250 µl cold PBS. The cell suspension was centrifuged at 350xg for 7 minutes at 4 ^{o}C. The supernatant was stored at -20 ^{o}C.

To enumerate osteoclasts, 1 x10^5 BM cells were seeded in quintuplicate in a 96-well flatbottom plate and stained using the tartrate-resistant acid phosphatase (TRAP) staining kit (Sigma Aldrich) according to the manufacturer's recommendations.

Antibodies for cell analysis

All antibodies used are described in table 1. Cells were analyzed on a Canto II FACS analyser with Diva software (BD Biosciences, Erebodegem, Belgium).

5-FU

5-Fluorouracil (5-FU, F6627, Sigma Aldrich) was dissolved in PBS and administered at a concentration of 150 mg/kg intraperitoneally. Cell recovery was determined every 2-3 days, but individual mice were only bled weekly to avoid excessive stress. A small volume of blood was drawn from the tail vein. Cell counts were performed on a Sysmex XP-300 counter. After lysis of erythrocytes, cells were stained with CD11b, Ly6G, BB20, CD3 and Ly6C-specific antibodies (table 1).

Quantitative PCR

After obtaining BM cells by flushing the femurs, the same femurs were flushed with PBS and RLT buffer (Qiagen) to obtain cell lysates of endosteal cells. RNA was obtained using the RNeasy mini kit (Qiagen) according to the manufacturer's recommendations and cDNA was generated using Superscript III (Invitrogen). Primer sets used for qPCR experiments are

shown in table 2. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed using TaqMan Universal Mastermix (Thermo Fischer) and Universal Probes (Roche) on a StepOnePlus cycler (Thermo Fischer). Relative gene expression was calculated using the comparative threshold cycle (C_T) method, with *Hprt*, *Abl* or *Gapdh* as the endogenous reference genes.

Administration of recombinant human G-CSF

Mice were injected intraperitoneally with 10 µg recombinant human G-CSF (Amgen, Thousand Oaks, California, USA) in 0.2 ml 0.1 % BSA/PBS once a day, for 3 consecutive days. Control mice received 0.2 ml 0.1% BSA/PBS.

Progenitor cell assays

Two hundred µl of PB was depleted of erythrocytes using a specific lysis buffer (LUMC Pharmacy). Next, the equivalent of 100 µl of PB was cultured in duplo in 3.5-cm dishes containing semisolid medium supplemented with recombinant murine GM-CSF (1.25 ng/ml; BD-Biosciences), recombinant murine IL-3 (25 ng/ml; BD-Biosciences), recombinant human Erythropoietin (0.2 units/ml; LUMC Pharmacy) and recombinant human G-CSF (100 ng/ml; Amgen). After 6 days of culture, the number of colonies (defined as an aggregate of ≥20 cells) was scored using an inverted light microscope.

Peripheral blood cell transplantations

Recipients were irradiated in Perspex chambers using an Orthovolt (Xstrahl Medical, Walsall, UK). A total dose of 9.5 Gy total body irradiation (TBI) was administered. Four hours

following TBI, 750 x10^3 PB mononuclear cells were injected via caudal vein injection in 200 µl of 0.1% BSA/PBS.

Osteoprotegerin and M-CSF

Recombinant murine osteoprotegerin (OPG) was obtained from R&D systems (Minneapolis, USA), dissolved in PBS and administered intravenously prior to G-CSF administration. OPG concentration was determined using a mouse OPG immunoassay (R&D systems) according to the manufacturer's recommendations. M-CSF concentrations were assessed using a mouse M-CSF ELISA (R&D Systems).

Extracellular vesicles

EV-depleted MSC medium was obtained by centrifuging MSC medium at 100,000xg at 4 °C for 16 hours using a Beckman Coulter Ultracentrifuge. MSC were cultured for 72 hours in EV-depleted medium. Culture supernatant was sequentially centrifuged at 350xg for 10 minutes and at 10,000xg for 30 minutes to discard cell debris. Supernatant was collected and centrifuged for 70 minutes at 100,000xg. The pellet containing EV was washed in PBS for 70 minutes at 100,000xg and resuspended in PBS. EV were quantified using a qNano particle analyzer (Izon Science, Oxford, UK). EV preparations had a mean particle diameter of 133.7 ± 3.2 nm. Typically, 5.3 x10^{10} ± 1.7 x10^{10} EV were isolated per 1 x10^6 MSC following 3 days of culture. Where indicated, EV were stained in Diluent C solution for 10 minutes using a PKH26 kit (Sigma-Aldrich). Staining was stopped by adding 1% BSA/PBS. Next, EV were washed for 70 minutes at 100,000xg and resuspended in PBS.

Statistical analysis

All values are presented as mean with standard error of the mean (SEM). All groups were compared using the unpaired t-test with Welch's correction when applicable. All statistical calculations were performed using GraphPad Prism software (La Jolla, California, USA). $P \leq 0.05$ was considered statistically significant.

Results

MSC administration increases hematopoietic stem –and progenitor cell cycle activity

To investigate the effect of MSC administration on the hematopoietic compartment in the BM, cohorts of C57BL/6 mice received 3 consecutive daily injections of MSC. On day 4, mice were sacrificed and BM cells were analyzed. The absolute number of HSC (defined as Lin^- $Sca\text{-}1^+c\text{-}Kit^{HI}CD34^-CD135^-$) was significantly decreased (figure 1 d), while the total number of white blood cells (WBC) per femur and the colony-forming capacity of the BM remained comparable to controls (figure 1 a, b). Moreover, there was a trend towards decreased numbers of LSK cells, HPC and MPP per femur (figure 1 c, e, f). To investigate whether the decrease in HSC numbers was due to altered cell cycle activity of HSPC, the cell cycle status of the hematopoietic cells after MSC administration was assessed. The frequency of LSK cells in the G1 phase of cell cycle was 3.2-fold increased compared to PBS-treated controls, while the frequencies of $Lin^-Sca\text{-}1^+c\text{-}Kit^{HI}$ (LSK) cells in G0 and the S/G2/M phase were decreased with 64% and 50.7% of PBS controls (figure 1g). A similar shift in cell cycle activity was observed for HSC and HPC/MPP (supplemental figure 1 a, b). The cytoreductive agent 5-FU kills actively cycling cells, including cycling HSPC and induces a BM stress response. In the PB, WBC were decreased within days after 5-FU injection (figure 1 h, i). Administration of MSC for 3 consecutive days followed by 5-FU injection delayed WBC recovery compared to controls receiving PBS and 5-FU. This delay was even more pronounced in the granulocytic compartment (figure 1 h, i). Together, these results indicate that administration of MSC leads to a reduction of the number of LSK cells in the BM and induces HSPC into cell cycle.

MSC downregulate niche factors in the BM

The hematopoietic stem cell niche regulates HSC cell cycle entry. Therefore, the observed increase in cell cycle activity of HSPC following MSC administration, may be explained by changes in the niche. Macrophages have been shown to contribute to anchoring HSC in the niche and their depletion leads to downregulation of HSC-retention factors including CXCL12 and VCAM in stromal cells and their depletion induced HSPC mobilization [14,15]. In turn, MSC act on cells of the innate immune system, including macrophages [22-24]. For these reasons, we hypothesized that MSC may alter the HSC niche through macrophages as intermediate cells. Therefore, the presence of osteomacs and $CD169^+$ macrophages was assessed in BM following MSC administration. A significant decrease in osteomacs and $CD169^+$ macrophages was observed, compared to PBS-injected controls (figure 2a-f). Moreover, osteoclasts, which are macrophages specialized in regulating bone metabolism, were also decreased ($p=0.057$, figure 2g). The decline in osteoclasts upon MSC administration coincided with increased levels of the osteoclast inhibitor osteoprotegerin (OPG) in the BM extracellular fluid ($p=0.07$), while the levels of M-CSF remained unchanged (supplemental figure 2a, c).

It has been reported that depletion of BM macrophages *in vivo* results in downregulation of *Cxcl12*, *Vcam, Ang-1* and *Scf* [13-15]. Similarly, following MSC administration, the expression of *Cxcl12* and *Vcam* was significantly decreased in endosteal cells, while a modest decrease in *Scf* expression was observed (figure 2h-j).

To further study the effect of macrophages on gene expression in stromal cells, *in vitro* culture experiments were performed in which cells of the immortalized macrophage cell line RAW264.7 were incubated with either S17 or MS-5 stromal cells. Cultures were performed in a transwell-setting, to investigate the effect of secreted factors or cell-cell contact. Next, gene expression was assessed. Direct cell-cell contact between RAW264.7 and stromal cells downregulated the expression of *Cxcl12*, *Vcam* and *Scf* significantly, compared to S17 and

MS-5 cultured in the absence of RAW264.7 cells. Factors secreted by RAW264.7 cells that were cultured in a transwell only mildly affected the expression of *Cxcl12*, *Vcam* and *Scf* (figure 2k-m).

Not only macrophages, but also B lymphocytes were significantly decreased in the BM and the peripheral blood following MSC administration (supplemental figure 2d-f). This decrease coincided with a significant reduction in *Il-7* expression in endosteal cells. Given the crucial role of IL-7 in B lymphopoiesis[25], these results suggest that MSC administration may impair B lymphopoiesis in the bone marrow.

MSC enhance G-CSF-induced HSPC mobilization

The depletion of macrophages and downregulation of *Cxcl12*, *Vcam* and *Scf* observed following MSC administration have been reported to also occur during G-CSF-induced HSPC mobilization [15,26]. Therefore, we hypothesized that MSC administration may affect G-CSF-induced HSPC mobilization. To this end, MSC were administered for 3 days to mice that were simultaneously mobilized with G-CSF. MSC and G-CSF co-injection induced a 2-fold increase in HSPC mobilization compared to G-CSF administration alone, while administration of MSC alone did not induce HSPC migration (figure 3a). This effect was specific for MSC since co-injection of splenocytes and G-CSF did not enhance HSPC mobilization (supplemental figure 3a). A modest increase in LSK cells was observed in the PB (figure 3b). To investigate whether MSC and G-CSF co-administration increased the number of long-term repopulating HSC in the PB, equal numbers of PB cells, obtained after co-injection of MSC and G-CSF or after G-CSF administration alone, were transplanted into lethally irradiated recipient mice. Recipients of PB obtained from MSC and G-CSF mobilized donors

showed significantly higher levels of donor leukocytes and granulocytes up to 19 weeks post-transplantation, compared to recipients of G-CSF-mobilized PB (figure 3c, d). This indicates that co-administration of MSC and G-CSF enhanced the mobilization of HSC with long-term repopulating ability compared to G-CSF alone.

Previously, it has been shown that the immunomodulatory capacity of MSC is enhanced in an inflammatory environment [24]. To examine whether exposure to inflammatory cytokines further enhances the capacity of MSC to increase G-CSF-induced HSPC mobilization, MSC were stimulated with IFN-γ and TNF-α prior to co-administration with G-CSF. IFN-γ and TNF-α-stimulated MSC indeed enhanced G-CSF mobilization, but cytokine-stimulated MSC did not further enhance this effect compared to unstimulated MSC (figure 3e).

The effect that MSC exert on the HSC niche seems to be independent of the effect established by G-CSF, since administration of MSC at 3 days prior to G-CSF administration induced the same enhancement of G-CSF-induced mobilization as simultaneous MSC and G-CSF administration (figure 3f). In addition, administration of MSC does not increase the levels of neutrophil elastase in the bone marrow (supplemental figure 3b; p= 0.28). A direct effect of G-CSF on MSC can be excluded, as MSC do not express the G-CSF receptor (supplemental figure 3c). Since osteoclasts were decreased upon MSC administration in combination with an increase in OPG (figure 2g and supplemental figure 2a), we assessed whether administration of OPG would enhance G-CSF-induced HSPC mobilization. However, no effect of OPG on G-CSF-induced mobilization was observed (supplemental figure 2b). To investigate the effect of MSC and G-CSF co-administration on niche genes, the expression of *Cxcl12*, *Vcam* and *Scf* was assessed in endosteal cells. As expected, the expression of these genes was decreased following G-CSF administration. Moreover, co-administration of MSC and G-CSF even further downregulated the expression of these genes (figure 3 g-i).

MSC enhance G-CSF-induced mobilization through a soluble factor

To investigate the fate of MSC upon intravenous administration, MSC transduced with a lentiviral construct containing *SFFV-DsR-Fluc* were administered for 3 days and visualized by luciferin. Upon intravenous administration, MSC migrated towards the lungs. No MSC migration to other locations was observed. This may be due to the sensitivity of the technique as a minimum of 5,000 MSC is required to obtain a signal that is distinguishable from background [20]. However, these results are in line with previous observations [19].
Since no MSC migration towards the BM was observed, we hypothesized that upon entrapment in the lungs, MSC secrete soluble factors that in turn affect the HSC niche and enhance G-CSF-induced HSPC mobilization. Therefore, MSC culture supernatant was administered to recipients that were simultaneously mobilized with G-CSF. Co-administration of MSC culture supernatant and G-CSF significantly enhanced G-CSF-induced mobilization, while administration of culture supernatant alone did not affect HSPC migration towards the PB (figure 3k).

MSC-derived extracellular vesicles enhance G-CSF induced HSPC mobilization

MSC have been reported to secrete extracellular vesicles (EV) [27]. To investigate whether EV are the supernatant-derived factor that enhanced G-CSF-induced HSPC mobilization, EV that were derived from 2 $x10^6$-0.2 $x10^6$ MSC were administered intravenously for 3 days to recipients that were simultaneously mobilized with G-CSF. Co-administration of EV and G-CSF induced HSPC mobilization at similar levels as co-injection of MSC and G-CSF (figure 4a). Moreover, administration of MSC-derived EV enhanced cell cycle activity of LSK cells and downregulated expression of *Cxcl12*, *Vcam* and *Scf* similar to MSC administration (figure 4 b-

e). Previously, it has been shown that MSC-derived EV migrate to the BM [28]. To investigate which BM cells were able to engulf MSC-derived EV, BM cells were incubated with PKH26-labeled EV for 4 hours and the $PKH26^{+}$ cells were identified. About 28% of the $CD45^{+}$ BM cells were able to engulf MSC-derived EV (figure 4f). Since >59% of the monocytic cells engulfed EV (figure 4f), we further investigated the phenotype of the EV^{POS} monocytic cells. EV^{POS} monocytic cells expressed F4/80, CD68 and MERTK at higher levels than EV^{NEG} monocytic cells. In addition, about 50% of the EV^{POS} cells expressed the M-CSF receptor (CD115; figure 4G-J). This indicates that macrophages are the main EV-engulfing population in the BM.

Discussion

MSC are a cellular component of the HSC niche and play a major role in the maintenance of HSC in the BM [1,2]. In addition, in an experimental transplantation model, co-administration of MSC and HSPC has a beneficial effect on HSC engraftment and hematopoietic recovery [18,19]. This suggests that MSC are capable of influencing the HSC niche, leading to changes that result in altered HSC homeostasis.

Here, we show that MSC administration indeed affects the HSC niche, as well as the BM hematopoietic compartment. Upon MSC administration, HSC numbers in the BM were decreased, coinciding with increased HSC cell cycling activity. Furthermore, MSC administration induced a decrease in BM macrophage subsets and concomitant downregulation of *Cxcl12*, *Vcam* and *Scf* expression in endosteal cells. Previous studies have shown that BM macrophages have a regulatory role in hematopoiesis and in the HSC niche [29]. Furthermore, depletion of osteal macrophages and a downregulation of *Cxcl12*, *Scf* and *Ang-1* mRNA is also observed during G-CSF-induced HSPC mobilization[15]. In steady state, macrophages regulate granulopoiesis and induce HSPC egress from the BM through circadian regulation of *Cxcl12* in stromal cells [30]. The decrease in Cxcl12 expression and HSPC egress is preceded by the downregulation of Liver X receptor(LXR)-target gene downregulation in macrophages[30]. Depletion of BM macrophages results in downregulation of *Cxcl12*, *Vcam* and *Scf*, increased HSC proliferation and HSPC mobilization [13-15,31]. Together, these and our data suggest that HSC-retaining factors in stromal cells are decreased due to macrophage depletion upon MSC administration and that increased HSPC cycling and mobilization may be a direct result of these events. This effect was specific for MSC since co-injection of splenocytes and G-CSF did not enhance HSPC mobilization.

To study the interaction between macrophages and stromal cells, we performed *in vitro* experiments in which RAW264.7 macrophages were co-cultured with stromal cells. Cell-cell contact between RAW264.7 and stromal cells downregulated *Cxcl12*, *Vcam* and *Scf* expression in stromal cells, while soluble factors secreted by RAW264.7 macrophages hardly influenced the expression of HSC-supporting genes. This further strenghtens the regulatory role of macrophages in the stem cell niche and indicates that cell-cell contact between stromal cells and macrophages is required for the downregulation of these factors.

When MSC were co-administered with G-CSF, HSPC mobilization was significantly enhanced, indicating that the HSC niche is alterered as a result of MSC injection. As these MSC are trapped in the lungs upon administration, it is conceivable that the observed increased mobilization is induced by a secreted factor. We considered that EV secreted by MSC could play such a role and therefore we embarked on experiments where we co-administered MSC or MSC-derived EV and G-CSF. Indeed, co-injection of EV and G-CSF induced HSPC mobilization at similar levels as MSC and G-CSF. Co-administration of MSC or MSC-derived EV and G-CSF further downregulated the expression of *Cxcl12*, *Vcam* and *Scf* in endosteal cells, compared to injection of either G-CSF or MSC alone. Moreover, the events induced by MSC administration are independent of the events induced by G-CSF, since sequential administration of MSC and G-CSF also enhanced HSPC mobilization.

We show that, *in vitro*, MSC-derived EV are engulfed by F4/80^{+}MERTK^{+}CD68^{+} BM-derived macrophages. Previously, it has been shown that CD68^{+} cells that express the G-CSF-receptor mediate G-CSF-induced HSPC mobilization [32]. We therefore propose that, *in vivo*, MSC-derived EV negatively affect this macrophage population, leading to downregulation of HSC retaining factors in the niche. This, in turn, induces a permissive state in the bone

marrow that allows for significantly enhanced HSPC mobilization when G-CSF is administered.

In recent years, studies have indicated that MSC-derived EV are associated with a variety of hematopoietic disorders [33-35]. MSC-derived EV are also thought to play a supporting role in tissue homeostasis and to influence responses to injury and infection [27,36]. EV, secreted by murine- or human MSC, are able to inhibit radiation-induced apoptosis of the murine hematopoietic cell line FDC-P1 [28]. In addition to this, administration of MSC-derived EV resulted in long-term survival in lethally irradiated mice due to a direct radioprotective effect on HSC [37].

In conclusion, both MSC and MSC-derived EV alter the stem cell niche and induce a permissive state in the BM. This state is characterized by macrophage depletion and downregulation of niche factors, thereby resulting in enhanced HSPC mobilization upon G-CSF administration. Further studies will be required to identify the exact EV-component(s) that are responsible for the effects on the stem cell niche. Identification of these factor(s) may potentially lead to novel HSPC mobilization strategies.

Acknowledgements

This work was financially supported by the Wijnand M. Pon foundation and by a grant from the Dutch government to the Netherlands Institute for Regenerative Medicine (NIRM, grant No. FES0908).

References

1. Morrison SJ, Scadden DT. The bone marrow niche for haematopoietic stem cells. *Nature*. 2014;505(7483):327-334.
2. van Pel M, Fibbe WE, Schepers K. The human and murine hematopoietic stem cell niches: are they comparable? *Ann N Y Acad Sci*. 2015.
3. Kunisaki Y, Bruns I, Scheiermann C, et al. Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature*. 2013;502(7473):637-643.
4. Mendez-Ferrer S, Michurina TV, Ferraro F, et al. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature*. 2010;466(7308):829-834.
5. Pinho S, Lacombe J, Hanoun M, et al. PDGFRalpha and CD51 mark human nestin+ sphere-forming mesenchymal stem cells capable of hematopoietic progenitor cell expansion. *J Exp Med*. 2013;210(7):1351-1367.
6. Zhou BO, Yue R, Murphy MM, Peyer JG, Morrison SJ. Leptin-receptor-expressing mesenchymal stromal cells represent the main source of bone formed by adult bone marrow. *Cell Stem Cell*. 2014;15(2):154-168.
7. Ding L, Morrison SJ. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature*. 2013;495(7440):231-235.
8. Ding L, Saunders TL, Enikolopov G, Morrison SJ. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature*. 2012;481(7382):457-462.
9. Winkler IG, Barbier V, Wadley R, Zannettino A, Williams S, Levesque JP. Positioning of bone marrow hematopoietic and stromal cells relative to blood flow in vivo: Serially reconstituting hematopoietic stem cells reside in distinct non-perfused niches. *Blood*. 2010;116(3):375-385.
10. Greenbaum A, Hsu YM, Day RB, et al. CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature*. 2013;495(7440):227-230.
11. Oguro H, Ding L, Morrison SJ. SLAM family markers resolve functionally distinct subpopulations of hematopoietic stem cells and multipotent progenitors. *Cell Stem Cell*. 2013;13(1):102-116.
12. Pruijt JF, van Kooyk Y, Figdor CG, Willemze R, Fibbe WE. Murine hematopoietic progenitor cells with colony-forming or radioprotective capacity lack expression of the beta 2-integrin LFA-1. *Blood*. 1999;93(1):107-112.
13. Chang MK, Raggatt LJ, Alexander KA, et al. Osteal tissue macrophages are intercalated throughout human and mouse bone lining tissues and regulate osteoblast function in vitro and in vivo. *J Immunol*. 2008;181(2):1232-1244.
14. Chow A, Lucas D, Hidalgo A, et al. Bone marrow CD169+ macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche. *J Exp Med*. 2011;208(2):261-271.
15. Winkler IG, Sims NA, Pettit AR, et al. Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs. *Blood*. 2010;116(23):4815-4828.
16. Kuiperij HB, van Pel M, de Rooij KE, Hoeben RC, Fibbe WE. Serpina1 (alpha1-AT) is synthesized in the osteoblastic stem cell niche. *Exp Hematol*. 2009;37(5):641-647.
17. Winkler IG, Hendy J, Coughlin P, Horvath A, Levesque JP. Serine protease inhibitors serpina1 and serpina3 are down-regulated in bone marrow during hematopoietic progenitor mobilization. *J Exp Med*. 2005;201(7):1077-1088.

18. van der Garde M, van Pel M, Millan Rivero JE, et al. Direct Comparison of Wharton's Jelly and Bone Marrow-Derived Mesenchymal Stromal Cells to Enhance Engraftment of Cord Blood CD34(+) Transplants. *Stem Cells Dev*. 2015;24(22):2649-2659.
19. Noort WA, Kruisselbrink AB, in't Anker PS, et al. Mesenchymal stem cells promote engraftment of human umbilical cord blood-derived CD34(+) cells in NOD/SCID mice. *Exp Hematol*. 2002;30(8):870-878.
20. Perez-Galarza J, Carlotti F, Rabelink MJ, et al. Optimizing reporter constructs for in vivo bioluminescence imaging of interferon-gamma stimulated mesenchymal stromal cells. *Exp Hematol*. 2014.
21. de Kruijf EJ, Hagoort H, Velders GA, Fibbe WE, van PM. Hematopoietic stem and progenitor cells are differentially mobilized depending on the duration of Flt3-ligand administration. *Haematologica*. 2010;95(7):1061-1067.
22. Melief SM, Schrama E, Brugman MH, et al. Multipotent stromal cells induce human regulatory T cells through a novel pathway involving skewing of monocytes toward anti-inflammatory macrophages. *Stem Cells*. 2013;31(9):1980-1991.
23. Maggini J, Mirkin G, Bognanni I, et al. Mouse bone marrow-derived mesenchymal stromal cells turn activated macrophages into a regulatory-like profile. *PLoS One*. 2010;5(2):e9252.
24. Bernardo ME, Fibbe WE. Mesenchymal stromal cells: sensors and switchers of inflammation. *Cell Stem Cell*. 2013;13(4):392-402.
25. Clark MR, Mandal M, Ochiai K, Singh H. Orchestrating B cell lymphopoiesis through interplay of IL-7 receptor and pre-B cell receptor signalling. *Nat Rev Immunol*. 2014;14(2):69-80.
26. Winkler IG, Pettit AR, Raggatt LJ, et al. Hematopoietic stem cell mobilizing agents G-CSF, cyclophosphamide or AMD3100 have distinct mechanisms of action on bone marrow HSC niches and bone formation. *Leukemia*. 2012;26(7):1594-1601.
27. Lai RC, Yeo RW, Lim SK. Mesenchymal stem cell exosomes. *Semin Cell Dev Biol*. 2015;40:82-88.
28. Wen S, Dooner M, Cheng Y, et al. Mesenchymal stromal cell-derived extracellular vesicles rescue radiation damage to murine marrow hematopoietic cells. *Leukemia*. 2016.
29. McCabe A, MacNamara KC. Macrophages: Key regulators of steady-state and demand-adapted hematopoiesis. *Exp Hematol*. 2016.
30. Casanova-Acebes M, Pitaval C, Weiss LA, et al. Rhythmic modulation of the hematopoietic niche through neutrophil clearance. *Cell*. 2013;153(5):1025-1035.
31. McCabe A, Zhang Y, Thai V, Jones M, Jordan MB, MacNamara KC. Macrophage-Lineage Cells Negatively Regulate the Hematopoietic Stem Cell Pool in Response to Interferon Gamma at Steady State and During Infection. *Stem Cells*. 2015;33(7):2294-2305.
32. Christopher MJ, Rao M, Liu F, Woloszynek JR, Link DC. Expression of the G-CSF receptor in monocytic cells is sufficient to mediate hematopoietic progenitor mobilization by G-CSF in mice. *J Exp Med*. 2011.
33. Roccaro AM, Sacco A, Maiso P, et al. BM mesenchymal stromal cell-derived exosomes facilitate multiple myeloma progression. *J Clin Invest*. 2013;123(4):1542-1555.
34. Muntion S, Ramos TL, Diez-Campelo M, et al. Microvesicles from Mesenchymal Stromal Cells Are Involved in HPC-Microenvironment Crosstalk in Myelodysplastic Patients. *PLoS One*. 2016;11(2):e0146722.

35. Viola S, Traer E, Huan J, et al. Alterations in acute myeloid leukaemia bone marrow stromal cell exosome content coincide with gains in tyrosine kinase inhibitor resistance. *Br J Haematol*. 2016;172(6):983-986.
36. Phinney DG, Pittenger MF. Concise Review: MSC-Derived Exosomes for Cell-Free Therapy. *Stem Cells*. 2017;35(4):851-858.
37. Schoefinius JS, Brunswig-Spickenheier B, Speiseder T, Krebs S, Just U, Lange C. Mesenchymal Stromal Cell-Derived Extracellular Vesicles Provide Long-Term Survival After Total Body Irradiation Without Additional Hematopoietic Stem Cell Support. *Stem Cells*. 2017.

Figure legends

Figure 1. MSC administration increases HSPC cell cycle activity. Following 3 days of intravenous MSC or PBS administration, femurs were isolated and analyzed for (A) total WBC numbers and (B) the number of colony-forming cells (CFU-C); n=6-8 per group. (C-F) The absolute number of $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{HI}CD34^{-}CD135^{-}$ (LSK), HSC, HPC and MPP per femur was determined by FACS analysis; n=6 per group. (G) Cell cycle activity of LSK cells was analyzed using a Ki67/DAPI staining. The frequencies of LSK cells in G0, G1 or S/G2/M was determined using flow cytometry. (H/I) Following 3 daily i.v. injections of MSC, mice received 5-FU at a dose of 150 mg/kg (day 0); WBC per ml of peripheral blood (H) and the absolute number of granulocytes per ml of peripheral blood (I) were determined at weekly intervals following 5-FU administration (n=5 per group). Data are depicted as mean ± SEM of 2 separate experiments. *p<0.05, **p<0.01, ***p<0.001 all compared to PBS.

Figure 2. MSC administration induces downregulation of niche factors in the bone marrow. (A-C) Osteomacs (n=12), (D-F) CD169+ macrophages (n=6) and (G) osteoclasts (n=10-13) were analyzed on day 4 after three consecutive days of PBS or MSC administration. Relative RNA expression for (H) *Cxcl12*, (I) *Vcam* and (J) *Scf* was determined in bone lining cells following PBS or MSC administration and depicted as relative expression compared to the household gene HPRT (n=11-15 from 5 separated experiments). K-M) Stromal cells downregulate (K) *Cxcl12*, (L) *Vcam* and (M) *Scf* upon cell-cell contact with RAW264.7 macrophages. RAW264.7 cells are co-cultured with S17 or MS-5 stromal cells either in a transwell (TW) or in direct cell-cell contact (Well; n=4-11 from 2-5 separated experiments). Data are depicted as mean ± SEM. *p<0.05, **p<0.005, ***p<0.0005 all compared to PBS.

Figure 3. MSC enhance G-CSF-induced HSPC mobilization through a soluble factor. (A) MSC (M) were administered intravenously for 3 days at a dose of 200 x10^3 cells per day to recipients that were simultaneously mobilized with G-CSF (G; 10 µg per day intraperitoneally for 3 days) or PBS (P) as a control (n= 16-30 per group). (B) The absolute number of $Lin^-Sca\text{-}1^+c\text{-}Kit^{HI}$ in the peripheral blood was analyzed using flowcytometry (n=8-11 per group). (C) Equal numbers of peripheral blood cells obtained from G-CSF or MSC + G-CSF mobilized donors were transplanted into lethally irradiated recipients and donor chimerism for (C) total leukocytes and (D) granulocytes was assessed (n=10 per group). (E) IFN-γ -and TNF-α-stimulated MSC enhance G-CSF mobilization at levels similar to unstimulated MSC. (F) MSC administration prior to G-CSF-induced mobilization significantly enhances HSPC mobilization. MSC were administered intravenously for 3 days at a dose of 200 x10^3 cells per day to recipients, followed by G-CSF administration on subsequent days (10 µg per day intraperitoneally for 2 or 3 days) or PBS as a control (n=3-6 from 2 independent experiments). (G-I) Relative RNA expression for (G*) Cxcl12,* (H) *Vcam* and (I) *Scf* was determined in bone lining cells following G-CSF or MSC + G-CSF administration and depicted as relative expression compared to the household gene HPRT (n=10-14 from 5 separated experiments). (J) MSC are trapped in the lung vasculature upon i.v. administration. Firefly-luciferase-transduced MSC were administered for 3 days. At day 4, MSC were visualized by administration of luciferin followed by bioluminescence imaging. (K) Simultaneous administration of G-CSF and serum-free culture supernatant (S) enhances G-CSF-induced HSPC mobilization (n= 5 per group). Mean ± SEM are depicted. *p<0.05 **p<0.01 ***p<0.001.

Figure 4. MSC-derived extracellular vesicles enhance G-CSF-induced mobilization. (A) MSC (M) or EV that were derived from 2 x10^6-0.2 x10^6 MSC were administered intravenously for 3 days to recipients that were simultaneously mobilized with G-CSF (G) or PBS (P) as a control. (n= 5-9 per group). (B) Cell cycle activity of LSK cells was analyzed using a Ki67/DAPI staining. The frequencies of LSK cells in G0, G1 or S/G2/M was determined and related to PBS controls; n=4-6 per group. (C-E) Relative RNA expression for (C) CXCL12, (D) VCAM and (E) SCF was determined in bone lining cells following MSC (M) or EV administration for 3 days to recipients that were simultaneously mobilized with G-CSF (G) or PBS (P) as a control. Gene expression is depicted as relative expression compared to the household gene HPRT (n=3-6). Significance is indicated compared to P. # p=0.055) (F) PKH26-labeled EV are primarily taken up by monocytes. The percentage of $CD45^+$ bone marrow cells, granulocytes (Gran), monocytes (Mon), B cells (B) and T cells (T) that have taken up EV are depicted within the total cell population. (G-H) Upon EV uptake, monocytes upregulate (G) F4/80, (H) CD68, (I) CD115 and (J) MERTK (data obtained from 1 experiment). EV^{neg} cells are depicted as a dotted line, EV^{pos} cells are depicted as filled histograms. Mean ± SEM are depicted. *p<0.05 **p<0.01 ***p<0.001.

Supplemental figure 1. MSC administration increases HSPC cell cycle activity. Following 3 days of intravenous MSC, femurs were isolated. Using a Ki67/DAPI staining, cell cycle activity of (A) HSC and (B) HPC/MPP was analyzed and related to PBS controls. Data are depicted as mean ± SEM, n=7 per group **p<0.01 compared to PBS.

Supplemental figure 2. Effect of MSC administration on the hematopoietic stem cell miroenvironment. Following 3 days of intravenous MSC or PBS administration (A) osteoprotegerin (OPG) levels were increased in bone marrow extracellular fluid upon MSC administration (n=3 per group). (B) OPG administration does not affect G-CSF-induced mobilization. OPG was administered at 10 µg or 1 µg per day for 3 days. At the same time points, PBS (P) or G-CSF (G) was administered; n=3-5 per group. (C) M-CSF (n=5-13) levels were determined in bone marrow extracellular fluid. Following 3 days of intravenous MSC or PBS administration B cells were significantly decreased in the (D) peripheral blood and (E) bone marrow (n=9-13). (F) This coincides with a decrease in *Il-7* expression in bone-lining cells. p<0.05, **p<0.01, ***p<0.001

Supplemental figure 3. (A) Splenocytes (S) were administered intravenously for 3 days at a dose of 200 x10^3 cells per day to recipients that were simultaneously mobilized with G-CSF (G; 10 µg per day intraperitoneally for 3 days) or PBS (P) as a control (n= 3 per group). Data are depicted as mean ± SEM. (B) Administration of MSC does not affect elastase levels in the bone marrow. Bone marrow extracellular extracts were obtained by flushing femurs with 250 µl cold PBS. The cell suspension was centrifuged at 2,300 g for 5 minutes and the supernatant was stored at -20 °C. Elastase activity was determined using the chromogenic substrate N-Succinyl-L-Ala-Ala-Ala-P-nitroanilide (Sigma, Zwijndrecht, The Netherlands).

Data are depicted as mean ± SEM (n=5-6) (C) *Csf3R* expression was assessed on peripheral blood cells (PB), bone marrow cells (BM) or MSC and depicted as relative expression compared to the household gene *Abl* (mean of triplicates are indicated).