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## **Biology of cytokine-induced hematopoietic stem and progenitor cell mobilization**

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### **Citation**

Kruijf, E. J. F. M. de. (2019, November 21). *Biology of cytokine-induced hematopoietic stem and progenitor cell mobilization*. Retrieved from <https://hdl.handle.net/1887/80691>

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**Author:** Kruijf, E.J.F.M. de

**Title:** Biology of cytokine-induced hematopoietic stem and progenitor cell mobilization

**Issue Date:** 2019-11-21

The image features two hands against a light beige background. The upper hand is positioned at the top left, with its index finger pointing downwards. The lower hand is positioned at the bottom right, with its index finger pointing upwards. Both hands have blood dripping from the tips of their index fingers. A thin horizontal line is visible across the middle of the image, separating the author's name from the title.

EVERT-JAN  
DE KRUIJF

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BIOLOGY OF  
CYTOKINE-  
INDUCED  
HEMATOPOIETIC  
STEM  
AND  
PROGENITOR  
CELL  
MOBILIZATION



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## ISBN

9789492303264

## BOOK DESIGN

Ilse Schrauwers, isontwerp.nl, Eindhoven

## COVER PHOTO

Erwin Olaf, Amsterdam

## PRINTING

Wihabo, Geffen

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# BIOLOGY OF CYTOKINE-INDUCED HEMATOPOIETIC STEM AND PROGENITOR CELL MOBILIZATION

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## PROEFSCHRIFT

ter verkrijging van  
de graad van Doctor aan de Universiteit Leiden,  
op gezag van Rector Magnificus prof. mr. C.J.J.M. Stolker,  
volgens besluit van het College voor Promoties  
te verdedigen op donderdag 21 november 2019 klokke 16.15 uur

door

EVERARDUS JOHANNES FRANCISCUS MARIA DE KRUIJF  
geboren te Gemert  
in 1965

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*Voor mijn vader  
Marinus E.M. de Kruijf (1939-1980)*



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# CHAPTER 1

## INTRODUCTION

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*“Blut ist ein ganz besondrer Saft”*

— Johann Wolfgang von Goethe, Faust I, verse 1740. —

# HEMATOPOIESIS

Every second, more than one million blood cells are produced in the human bone marrow to replenish peripheral blood cells that have been destroyed or have come to the end of their lifespan.<sup>1</sup> The majority of these cells derive from hematopoietic stem cells (HSC), which reside in the bone marrow microenvironment, where they are sheltered from external influences. Till and McCulloch were first to demonstrate the existence of these multipotent stem cells in mice.<sup>2</sup> These researchers accidentally observed that intravenously injection of bone marrow cells into previously irradiated mice led to the formation of colonies of proliferating cells in the animals' spleens.<sup>2</sup> These colonies, termed colony-forming unit-spleen cells (CFU-S), appeared to consist of differentiated blood cells of multiple lineages and a subset of these colonies could again form CFU-S when transplanted into secondary hosts.<sup>3</sup> Following these and other experiments conducted during the 1960s and 1970s, HSC were defined as single cells with a lifelong ability to self-renew and to differentiate into all blood cell lineages.<sup>4,5</sup> The unique properties of HSC are demonstrated by the observation that a single HSC is able to reconstitute lifelong multilineage hematopoiesis following transplantation into lethally irradiated mice.<sup>6</sup>

## HEMATOPOIETIC STEM AND PROGENITOR CELLS

Much of our understanding of hematopoiesis comes from the study of mice, since they have always been more amenable to functional *in vivo* assays than humans. In the murine and the human hematopoietic systems, HSC reside at the top of the hematopoietic hierarchy and give rise to the functional effector cells of at least nine distinct cell types (shown in **Figure 1**). In mice, long-term HSC (LT-HSC) have historically been defined as cells enabling repopulation beyond 12 weeks after transplantation. Cells that generate all lineages but extinguish before 12 weeks after transplantation are termed short-term HSC (ST-HSC).<sup>7</sup> A subgroup of HSC, named intermediate-term HSC (IT-HSC), is capable of engraftment beyond 12 weeks but becomes extinct between 6-8 months.<sup>8</sup> In humans, the heterogeneity of HSC is less understood. All differentiated cells are produced from HSC in successive differentiation processes of increasingly committed hematopoietic progenitor cells (HPC).<sup>7,9</sup> The immediate progeny of HSC are multipotent progenitor cells (MPP), which retain full lineage potential but have a limited capacity for self-renewal. Multipotent progenitors give rise to oligopotent progenitors, including the common lymphoid progenitor (CLP) in mice, the multilymphoid progenitor (MLP) in humans and the common myeloid progenitor (CMP) in both mice and humans, which possess more restricted differentiation potential.<sup>7</sup> These oligopotent progenitors subsequently give rise to lineage-restricted progenitors, from which all of the mature blood cells ultimately derive. This hierarchical model presumes that, during this process of

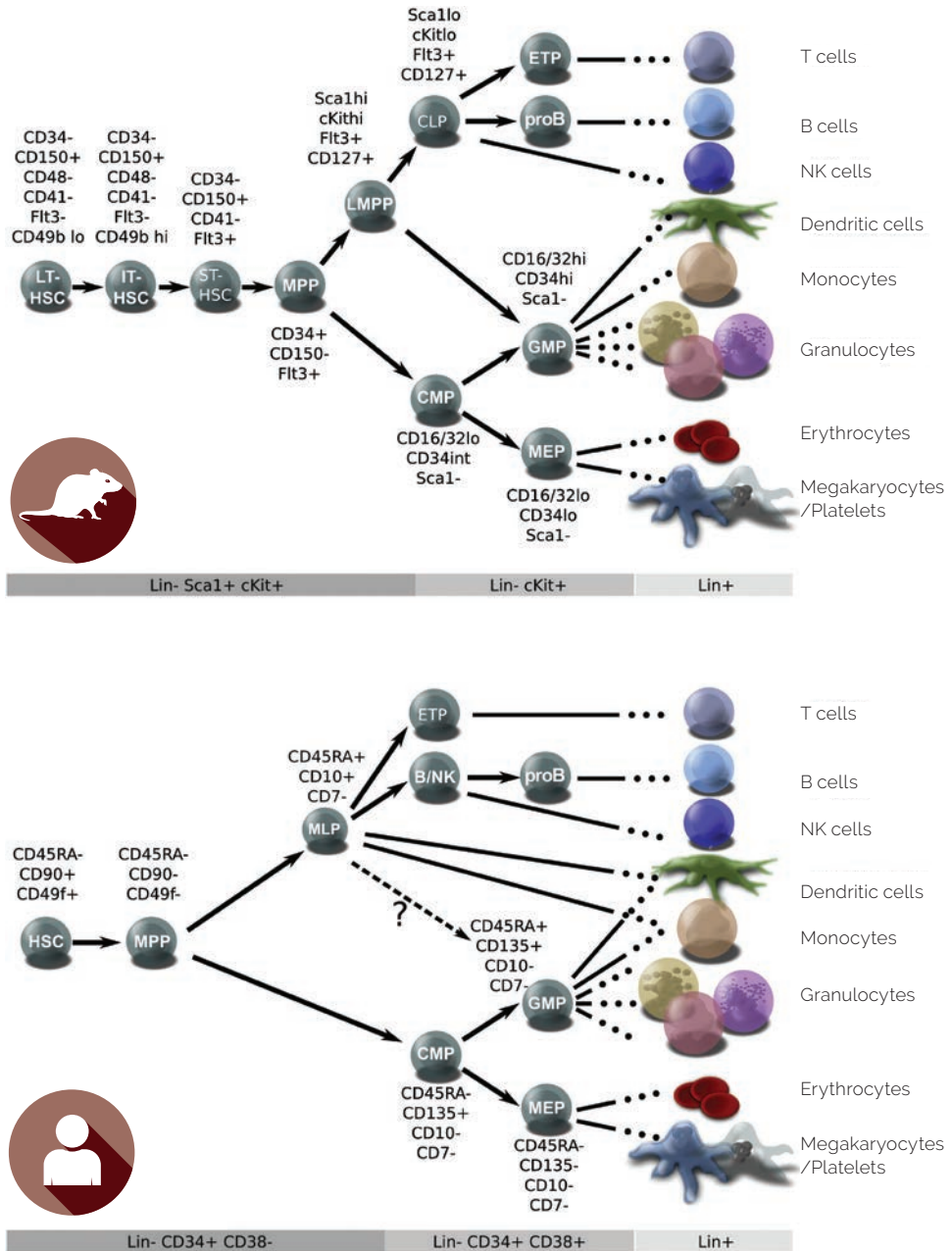


**FIGURE 1.** Hierarchical model of lineage determination in murine and human hematopoiesis.

HSC and their multi- and oligopotent progenitors are depicted in gray with corresponding surface markers. Terminally differentiated cells are shown on the right. (From: Doulatov S. et al., *Cell Stem Cell* 2012; 10:120-136.)

differentiation, the proliferative potential of the hematopoietic cells is gradually lost. Furthermore, the ability for self-renewal is only assigned to the most primitive, "true" stem cells at the top of the hierarchy. Many data support this model, which, however, has been challenged. Rather than differentiate in a strict hierarchy, hematopoietic stem and progenitor cells (HSPC) may do so in a reversible continuum, in which it is possible for the phenotype of the primitive cells to change from engraftable stem cells to progenitors and back.<sup>10</sup> In addition, recent data indicate that in adult human bone marrow, differentiated cell populations can branch directly from multipotent progenitors with few intervening oligopotent intermediates.<sup>11</sup>

Hematopoietic stem cells, oligopotent –and multipotent progenitor cells have the same morphologic characteristics. Therefore, the characterization of these different cell populations must be achieved by sophisticated techniques, broadly categorized here by the cell type that is being measured and the experimental conditions that are used.

## HEMATOPOIETIC STEM CELLS - SURFACE MARKERS

Monoclonal antibodies targeted against surface markers expressed on hematopoietic cells can be used to dissect the different cell types in the hematopoietic system (shown in **Figure 1**).

In an adult mouse, functional HSC are found in a subset of bone marrow cells that does not express cell-surface markers present on lineage (Lin)-committed hematopoietic cells but does express high levels of stem cell antigen-1 (Sca-1) and the c-Kit receptor (cluster differentiation (CD) 117).<sup>12</sup> This population of Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> (LSK) cells comprises around 0.1% of the total bone marrow cells.<sup>13</sup> In some, but not all, mouse strains, the presence of Thy-1 (CD90) can also be used as an additional cell surface marker to identify functional HSC.<sup>14</sup> Cells in this "KTLS" population were shown to be the only cells in murine bone marrow that can give rise to long-term multilineage repopulation in syngeneic and allogeneic transplantation.<sup>15</sup>

The CD2/signaling lymphocyte activation molecule (SLAM) family of receptors has been shown to further phenotypically differentiate HSPC.<sup>16</sup> The cell surface receptors CD150 and CD244 and its ligand CD48 can be used to reliably differentiate between highly purified HSC (CD150<sup>+</sup>/CD244<sup>-</sup>/CD48<sup>-</sup>), multipotent progenitors (CD150<sup>-</sup>/CD244<sup>+</sup>/CD48<sup>-</sup>) and oligopotent progenitors (CD150<sup>-</sup>/CD244<sup>+</sup>/CD48<sup>+</sup>).<sup>16,17</sup> Using the expression of the SLAM receptor members CD48 and CD150 in combination with LSK, CD34 and CD135, LSK cells can be further subdivided into dormant and activated HSC and different MPP subsets (MMP1, -2 and -3).<sup>12</sup>

The adhesion receptor CD97 is a member of the epidermal growth factor-seven transmembrane (EGF-TM7) family of adhesion receptors and is differentially expressed on HSPC. It can therefore be used to differentiate stem and progenitor cell populations, as HSC activity is mainly found in the CD97-intermediate

population of murine bone marrow cells.<sup>18</sup>

Micro-array analysis of the genes expressed by murine HSC demonstrated increased expression of the gene encoding the murine endothelial protein C receptor (EPCR).<sup>19</sup> The expression of EPCR on the surface of transplanted bone marrow cells is positively correlated with long-term hematopoietic reconstitution activity and, in combination with other surface markers (EPCR<sup>+</sup>/CD150<sup>+</sup>/CD48<sup>-</sup>/CD45<sup>-</sup>), significantly enriches murine HSC.<sup>19,20</sup>

Alternatively, staining murine bone marrow cells with specific dyes can be used to dissect populations of HSPC. Intracellular staining with the vital dye Hoechst 33342 allows for delineating a specific population of cells called side population (SP) cells.<sup>21</sup> These SP cells were shown to contain the majority of bone marrow HSC in competitive repopulation assays and to be enriched at least 1,000-fold for *in vivo* reconstitution activity compared to total bone marrow.<sup>21</sup> In combination with the SLAM marker CD150, SP cells can be further divided into functionally different populations of long-term repopulating cells.<sup>22</sup> Rhodamine-123 (Rho) is a fluorescent dye that accumulates intracellularly and binds to mitochondria with increasing intensity as cells become activated.<sup>23,24</sup> Cells with less active mitochondria exhibit a reduced uptake of Rho. Rho staining of HSPC allows for the separation of Rho<sup>+</sup>(dull) cells with long-term repopulating capacity and Rho<sup>++</sup>(bright) cells, representing HSC and HPC, respectively.<sup>23,24</sup>

Markers that are applicable in murine hematopoiesis are not necessarily useful in humans, e.g. murine HSC express the surface marker CD34 at low to negative levels, whereas human HSC are CD34<sup>+</sup>.<sup>25-27</sup> However, human bone marrow CD34<sup>+</sup> cells are heterogeneous, since this population not only contains HSC but also differentiated progenitors.<sup>28</sup> For clinical purposes, CD34 is widely used as a cell surface marker to identify and isolate human HSPC because the number of CD34<sup>+</sup> cells in a hematopoietic cell transplant correlates with the rate of hematopoietic reconstitution.<sup>29</sup>

The purification of human HSC requires the exclusion of mature lineage markers (Lin<sup>-</sup>) such as CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b and GlyA.<sup>30</sup> The use of additional surface markers, such as CD38, CD45RA, Thy1 and integrin  $\alpha$ 6 (ITGA6, CD49f), can further enrich a cell population for HSC.<sup>30,31</sup> This improvement is illustrated by the observation that in an experimental setting, a single Thy1<sup>+</sup>Rho<sup>lo</sup>CD49f<sup>+</sup> human bone marrow cell is capable of long term multilineage engraftment.<sup>31</sup>

Interestingly, most markers used for the identification of HSC do not contribute to their functionality, suggesting that new, more relevant markers may still be awaiting discovery.<sup>32</sup>

## HEMATOPOIETIC STEM & PROGENITOR CELLS - *IN VITRO* ASSAYS

Hematopoietic progenitor cells can be assessed by *in vitro* colony-forming cell (CFC) assays since HPC are capable of forming colonies in semisolid culture mediums, such as methylcellulose, in the presence of relevant hematopoietic growth factors and under appropriate temperature and CO<sub>2</sub> conditions.<sup>33,34</sup> CFCs mostly consist of lineage-restricted colonies such as erythroid-restricted burst-forming units-erythroid (BFU-E), which are more immature than the colony-forming units-erythroid (CFU-E); megakaryocyte-restricted colony-forming units (CFU-Mk); colony-forming units-granulocytes (CFU-G), colony-forming units-monocytes/macrophages (CFU-M); and colony-forming units-granulocytes/macrophages (CFU-GM).<sup>35,36</sup> The most immature (multipotent) CFC measurable contains granulocytes, erythrocytes, macrophages and often megakaryocytes (CFU-GEMM) and is usually measured at day 12 after culture initiation (Figure 1).<sup>36</sup> However, these short-term assays do not detect HSC, as the lifespan of the progenitor cells in these assays is limited to a maximum of 2-3 weeks, which is insufficient to allow HSC to produce differentiated progeny.<sup>17</sup>

To assess the frequency of HSC, the cobblestone area-forming cell (CAFC) assay and the long-term culture-initiating cell (LTC-IC) assay were introduced in the 1980s.<sup>37-39</sup> Both of these liquid culture assays use feeder cells to provide the substrate and regulatory growth factors required for the growth of HSC. In the CAFC assay, the stromal cell layers are inoculated with hematopoietic cells. Different hematopoietic cell subsets form colonies in the adherent stromal layer that look like flattened, optically dense cells with a cobblestone-like appearance.<sup>37,40</sup> Ploemacher et al. have developed a quantitative CAFC assay by using limited dilution and Poisson statistics to assess the frequency of HSPC.<sup>37,40</sup> In this assay, day 7 CAFC correlate with HPC, whereas day 28 CAFC correlate with HSC that show long-term *in vivo* repopulating capacity.<sup>37,40</sup> The disadvantages of both systems are that they are time-consuming and difficult to standardize.<sup>41</sup> Originally developed to study murine hematopoiesis, both assays have been adapted to also examine human hematopoiesis.<sup>42,43</sup>

## HEMATOPOIETIC STEM & PROGENITOR CELLS - *IN VIVO* ASSAYS

Stem cells are defined by their long-term hematopoiesis reconstitution ability upon transplantation into a lethally-irradiated host. Therefore, transplantation remains the single functional assay to assess the presence and frequency of stem cells.

Primary transplantation requires (sub)lethal total body irradiation of the recipients, followed by the transplantation of donor-derived hematopoietic cells. At different time points post-transplantation, the survival of the recipients is assessed and the presence of donor peripheral blood cells is measured. Short-term recipient survival ("radio-protection") at 4-5 weeks post-transplantation demonstrates the

short-term repopulating ability of HPC. Multilineage reconstitution at 12 weeks after transplantation is used to demonstrate long-term multilineage repopulating ability of HSC.<sup>17,44</sup>

Transplantation assays can be either competitive or non-competitive. The competitive repopulation unit (CRU) assay measures the functional potential of an unknown source of HSC against a pre-set, known number of HSC.<sup>36</sup> The tested cell population is transplanted into irradiated recipients along with  $1-2 \times 10^5$  competing bone marrow cells. These competing cells are preferably obtained from a congenic animal with an identifiable phenotype that is distinct from both the donor and the recipient, such as the Ly5.1/Ly5.2 polymorphism in C57BL/6 mice.<sup>17</sup> These cells provide short-term radioprotection and make the quantification of the HSC frequency possible if limiting dilutions of test cells are transplanted.<sup>45</sup> However, the reliability of the CRU-assay is critically dependent on the number of HSC present in the populations that are assessed; when too few ( $<1 \times 10^5$  recipient cells) or too many ( $>2 \times 10^7$  recipient cells) HSC are present, the data may be less reliable.<sup>36,46</sup> Another limitation is its dependence upon homing and engraftment processes, which may be disturbed without altering stem cell function per se.<sup>36</sup> However, this limitation might be overcome by the direct injection of hematopoietic cells into the mouse's bone marrow (intra-bone marrow injection), circumventing the potential influence of factors interfering with the homing of HSC.<sup>47,48</sup>

In a non-competitive transplantation assay, hematopoietic cells are transplanted into lethally irradiated congenic recipients without competitor cells; this process more closely resembles clinical transplants in an autologous and allogeneic setting.<sup>17</sup>

The ultimate indicator for HSC activity is the secondary transplantation assay. In this assay, at 3-6 months after the first transplantation, the bone marrow of the primary recipients is transplanted into the secondary recipients using a distinguishable marker between the primary donor and the secondary recipient. At three months post-secondary transplantation, the presence of donor-derived hematopoiesis confirms the presence of repopulating stem cells in the primary recipient's bone marrow.<sup>17</sup>

Human HSC are studied by transplantation of human hematopoietic cells into immune-deficient mice.<sup>49</sup> These mice lack a functional immune system (B- and T-cells) and are, therefore, unable to reject human cells. Human hematopoietic cells can be successfully transplanted in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice and establish long-term multilineage repopulation through the engraftment of multipotent CD34<sup>+</sup>/CD38<sup>-</sup> cells, called SCID-repopulating cells (SRC).<sup>50</sup> In NOD/SCID mice, natural killer (NK) cells remain active and are able to resist engraftment. Therefore, other immunodeficient mouse strains have been developed to address this problem. Truncation (NOG-mouse) or deletion in the IL-2R- $\gamma$ -chain (NSG-mouse) results in the complete loss of B, T

and NK cells.<sup>51</sup> NSG mice engrafted with human HSC show significantly increased numbers of human CD45<sup>+</sup> cells in the host bone marrow in comparison with similarly treated NOD/SCID mice.<sup>51,52</sup> Newer generations of humanized mice, e.g. with strains that possess knock-in alleles for human cytokines (such as macrophage colony-stimulating factor (M-CSF) or thrombopoietin (TPO)), are currently under development in order to further study human HSC biology.<sup>53</sup>

## THE HEMATOPOIETIC STEM CELL NICHE

During fetal development, HSC expand in the fetal liver during the same time that the cartilage and bone are created by chondrocytes and osteoblasts within mesenchymal condensations.<sup>54</sup> The bone that is formed at this stage will become vascularized, which allows for the homing of HSC and the colonization of the fetal bone marrow.<sup>54</sup> The adult bone marrow is a highly vascularized tissue, with arterioles transitioning to venous sinusoids in close proximity to the endosteum.<sup>55</sup> During homeostasis, the vast majority of HSC reside in the bone marrow, with only a small minority of HSC present in the circulation.<sup>56</sup> In the mid-1970s, it was observed that HSC are not evenly distributed within the bone marrow, with more primitive cells localized towards the endosteal areas.<sup>57</sup> It also became apparent that culturing of HSC requires the presence of a bone marrow stromal feeder layer. Based on these findings and on his own experiments in c-Kit-mutated mice, Schofield proposed the concept of a specific stem cell niche within the bone marrow.<sup>58,59</sup> In this concept, the HSC niche is a specific micro-environment in the bone marrow, where the HSC are in close contact with and supported by other cells that determine its behavior, sustain its continued proliferation as a stem cell and prevent its maturation.<sup>58</sup>

The current understanding of the HSC niche is still incomplete, as it appears to be a complex ecological system composed of different cellular players, including osteoprogenitors, osteoblastic cells, vascular endothelial cells, mesenchymal stromal cells, neuronal cells and hematopoietic cells, such as macrophages and megakaryocytes, each with their own specialized functions (shown in **Figure 2**).<sup>60-64</sup> It has been postulated that functionally different HSC niches coexist within the bone marrow, although thus far this has not been sufficiently substantiated by the available, often conflicting, data.<sup>60,65,66</sup>

A direct role for osteoblasts in HSC support was initially suggested by experiments in which the manipulation of osteoblast numbers, either pharmacologically or genetically, correlates with the number of HSC in the bone marrow.<sup>67,68</sup> In irradiated mice, transplanted HSC seem to preferentially locate near the endosteal surfaces of the trabecular bone, whereas they are randomly distributed in nonirradiated recipients.<sup>69,70</sup> Later studies suggested that the role of osteoblastic cells in the HSC niche is more indirect than direct as neither the depletion nor the expansion of osteoblasts has an immediate effect on HSC frequency.<sup>71-74</sup> Also, recent *in vivo*

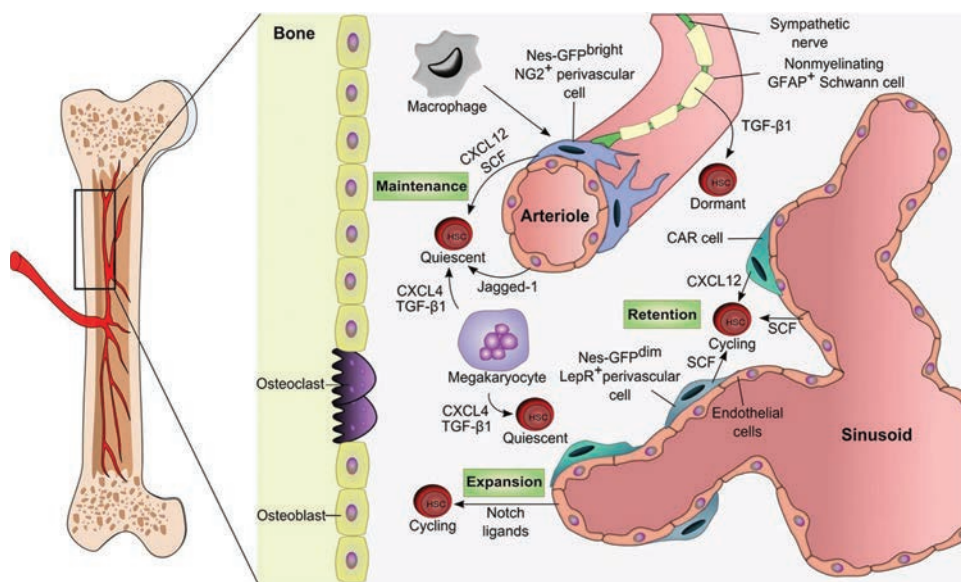


**FIGURE 2. The adult bone marrow HSC niche.**

Non-cycling HSC are found around arterioles where factors such as CXCL12 and SCF, which are secreted by perivascular, endothelial, Schwann and sympathetic neuronal cells, promote their maintenance. Activated HSC are located near sinusoidal niches, which are likely diverse in their influence for self-renewal, proliferation and differentiation. Hematopoietic cells such as macrophages and megakaryocytes are cells of hematopoietic origin that maintain the HSC niche. (From: Boulais PE, Frenette PS, *Blood* 2014; 125:2621-2629.)

imaging studies do not reveal a significant spatial correlation between HSC and osteoblastic cells.<sup>69,71,75–77</sup> Additionally, the deletion of HSC-supportive factors such as stem cell factor (SCF) or CXCL12 (stromal cell-derived factor 1, SDF-1) in osteoblastic cells does not alter HSC numbers, but instead results in the loss of lymphoid progenitors in the bone marrow.<sup>78,79</sup> These data support the current understanding that mature osteoblasts might play a direct role in lymphopoiesis but only have an indirect role in modulating HSC.

## THE PERIVASCULAR NICHE

In recent years, attention has focused on the role of perivascular cells in the formation of the HSC niche, most importantly mesenchymal stromal cells (MSC) and endothelial cells.

Mesenchymal stromal cells are a heterogeneous population of cells, capable of differentiating into adipocytes, chondrocytes, fibroblasts and osteoblasts.<sup>80,81</sup> In the perivascular niche, MSC can be distinguished from other cells by the expression of CD51 and the platelet-derived growth factor receptor alpha (PDGFRA or CD140a).<sup>82</sup>

Additionally, these perivascular MSC differentially express one or more of the following markers: neuron/glia antigen 2 (NG2), leptin receptor (LEPR) and nestin, an intermediate filament protein known as a marker for neural stem cells.<sup>83-85</sup> NG2<sup>+</sup>/LEPR<sup>+</sup>/nes<sup>bright</sup> MSC are generally located around arterioles, close to the endosteum, and NG2<sup>+</sup>/LEPR<sup>+</sup>/nes<sup>dim</sup> reticular cells are mostly situated around the sinusoids, away from the endosteal surface.<sup>83</sup> Hematopoietic stem cells commonly co-localize with nestin-GFP transgene (Nes-GFP)<sup>+</sup> cells, suggesting that HSC prefer to localize close to arterioles.<sup>83,84</sup>

Perivascular cells, which express CXCL12, a chemokine responsible for the retention of HSC in the bone marrow, co-localize with HSC adjacent to sinusoids.<sup>76</sup> These CXCL12-abundant reticular (CAR) cells might overlap in function with LEPR<sup>+</sup> perivascular cells, which also express CXCL12.<sup>76,78,85</sup> Deep confocal imaging studies indicate that nearly all HSC co-locate with LEPR<sup>+</sup> and CXCL12<sup>high</sup> cells, indicating the essential role of these cells in the niche.<sup>77</sup> LEPR<sup>+</sup> perivascular cells are a major source of SCF in the bone marrow, as was shown in experiments where SCF was either overexpressed or conditionally deleted in a transgenic mouse model.<sup>86</sup> Stem cell factor and CXCL12 are also produced by vascular endothelial cells, which line the surface of blood vessels. The conditional deletion of SCF in endothelial cells leads to the depletion of HSC in the bone marrow.<sup>86</sup> When CXCL12 is conditionally deleted from endothelial cells, HSC are depleted but not mobilized, which is probably related to the fact that CXCL12 is approximately 100-fold less expressed in endothelial cells as opposed to perivascular MSC.<sup>78,79</sup> Endothelial cells also express various Notch ligands, which inhibit excessive HSC differentiation, and endothelial-leukocyte adhesion molecule 1 (E-selectin), which is implicated in promoting HSC proliferation.<sup>87-89</sup>

In the perivascular niche, Nes-GFP<sup>+</sup> cells are closely associated with sympathetic nerve fibers and express the  $\beta$ 3-adrenergic receptor.<sup>84</sup> Circadian noradrenaline secretion by the sympathetic nervous system (SNS) decreases CXCL12 expression in these cells, resulting in a rhythmic release of HSC from the bone marrow niche and their subsequent mobilization into the bloodstream.<sup>84,90</sup> Sympathetic nerve fibers are sheathed by non-myelinating Schwann cells that are Nes<sup>+</sup>, express HSC niche factor genes (e.g. *Cxcl12* and *Scf*) and co-localize with a significant proportion (around 25%) of HSC in the bone marrow, further supporting the important role of the SNS in regulating the HSC niche.<sup>91</sup> Damage to the SNS from neurotoxic chemotherapy results in impaired hematopoietic regeneration, due to the selective loss of adrenergic innervation.<sup>92</sup> Interesting from a clinical standpoint is the finding that neuroprotection in mice treated with chemotherapy can rescue bone marrow engraftment and mobilization.<sup>92,93</sup>

Together, these data support the hypothesis that perivascular niches dictate HSC maintenance and quiescence in the bone marrow.

## HEMATOPOIETIC CELLS SUPPORTING THE NICHE

The progeny of HSC, such as macrophages and megakaryocytes, also constitute important feedback regulators within the perivascular niche. Macrophages indirectly support HSC by influencing the activity of other bone marrow niche cells.<sup>94-96</sup> Several macrophage populations in the bone marrow have been described in terms of their surface antigen expression, location and function.<sup>96</sup> Significant overlap between these populations remains, making it difficult to distinguish between various bone marrow macrophage subtypes and their respective functions in supporting HSC and hematopoiesis. Bone marrow macrophages mostly express the surface proteins CD68 (microsialin), CD106 (vascular cell adhesion molecule-1, VCAM-1), CD169 (sialoadhesin), variable levels of CD11b, and F4/80 and EMR1 in mice and humans, respectively.<sup>96,97</sup>

Osteal tissue macrophages (osteomacs) are F4/80<sup>+</sup> cells that regulate osteoblast function by forming a canopy over bone-lining osteoblasts.<sup>98</sup> The *in vivo* depletion of osteomacs, in either macrophage Fas-induced apoptosis (Mafia) transgenic mice or in wild-type mice by administration of clodronate-loaded liposomes, is associated with the loss of endosteal osteoblasts, the significant reduction of HSC-trophic cytokines (e.g. CXCL12, SCF, angiopoietin 1 (Ang-1) and osteocalcin) and the migration of HSC to the peripheral blood.<sup>94</sup>

CD169<sup>+</sup> macrophages were identified as critical stromal niche supportive cells that indirectly regulate HSC cycling and HSC pool size.<sup>95,99</sup> Using macrophage-specific conditional depletion models, reductions in bone marrow CD169<sup>+</sup> macrophage numbers were shown to lead to reduced CXCL12 levels, the selective down-regulation of HSC retention genes (*Cxcl12*, *Angpt1*, *Scf* and *Vcam1*) in Nes<sup>+</sup> niche cells and the migration of HSPC to the bloodstream.<sup>95</sup> Under steady state conditions, the depletion of bone marrow resident macrophages increases both HSC proliferation and the absolute number of quiescent HSC.<sup>99</sup> Furthermore, CD169<sup>+</sup> macrophages are essential for supporting erythropoiesis by forming erythroblastic islands, where a central macrophage is surrounded by erythroid precursors in varying stages of development.<sup>100</sup> The depletion of these macrophages, as a consequence of granulocyte colony-stimulating factor (G-CSF) or fms-like tyrosine kinase 3 (Flt3) ligand (FL) administration, leads to a transient decrease in intramedullary erythropoiesis.<sup>101,102</sup> In addition, CD169<sup>+</sup> macrophages are essential to the circadian fluctuation in circulating HSC, as they are required for the clearance of aged (CD62<sup>lo</sup>/CXCR4<sup>hi</sup>) neutrophils, which in turn results in decreased CXCL12 expression and subsequent HSC mobilization.<sup>103</sup>

Megakaryocytes (MGK), which are the precursors of thrombocytes, are often closely associated with sinusoidal endothelium in the bone marrow by extending cytoplasmic protrusions into the sinusoids.<sup>61,63</sup> Interestingly, around 20% of phenotypic HSC co-localize with MGK.<sup>63</sup> Megakaryocytes play a role in HSC

maintenance by producing CXCL4 (or platelet factor 4), transforming growth factor beta-1 (TGF- $\beta$ 1) and TPO.<sup>104-106</sup> The depletion of MGK results in the activation of quiescent HSC and increased HSC proliferation through reduced levels of biologically active TGF- $\beta$ 1 in the bone marrow, suggesting a supportive role of MGK in the HSC niche (**Figure 2**).<sup>104,105</sup>

## STEM CELL MOBILIZATION

Under steady state conditions, the peripheral blood contains only very small numbers of HSPC. Hematopoietic stem and progenitor cells, which reside in the bone marrow, can be induced to exit the bone marrow and migrate towards the peripheral blood; this process is called mobilization. In a clinical setting, this phenomenon is utilized to harvest these HSPC from the peripheral blood to allow for subsequent therapeutic purposes.

The mobilization of HSPC was first described in 1977 when there was a fourfold increase of CFU-GM in the peripheral blood of healthy volunteers after the administration of endotoxin.<sup>107</sup> It was subsequently found that CFU-GM were also increased in the recovery phase after chemotherapy.<sup>108</sup> Thereafter, a wide variety of hematopoietic growth factors, chemokines and other molecules have been identified as being capable of inducing HSPC mobilization. Nowadays, several agents have been approved for HSPC mobilization in a clinical setting, including G-CSF, granulocyte-macrophage colony-stimulating factor (GM-CSF), SCF and plerixafor (or AMD3100).<sup>109</sup> Others, such as interleukin-1 (IL-1), interleukin-8 (IL-8), anti-very late antigen 4 (anti-VLA-4) or Flt3-ligand (FL), are mainly used in experimental animal studies. The mobilizing agents G-CSF, IL-8 and FL, which are used in the studies described in this thesis, will be described below in more detail.

### GRANULOCYTE COLONY-STIMULATING FACTOR (G-CSF)

It took over 20 years before the discovery of hematopoietic growth factors led to the clinical application of G-CSF (also known as colony-stimulating factor 3, CSF3) in HSPC mobilization.<sup>110</sup> In the 1960s, it was discovered that expanding colonies of granulocytes and monocytes requires the presence of the then-unknown factors that are now called colony-stimulating factors (CSF).<sup>111,112</sup> Depending on the type of colony growth that was stimulated, four different CSF subtypes could be identified, including GM-CSF that stimulated granulocyte and macrophage colony growth, M-CSF for the induction of macrophage colonies, G-CSF for granulocyte colonies and multi-CSF (later designated IL-3) for a broad range of hematopoietic cell colonies.<sup>112</sup> In 1983, the purification of murine G-CSF was established, which was followed the next year by the purification of human G-CSF.<sup>113,114</sup>

Granulocyte colony-stimulating factor primarily affects hematopoietic cells of the myeloid lineage by stimulating the proliferation and differentiation of these cells.<sup>115</sup> After binding to its receptor, G-CSF activates the phosphorylation of the Ras/MAP kinase pathway and the protein tyrosine kinases JAK1 and JAK2.<sup>116,117</sup> In granulocytes, G-CSF enhances chemotaxis and superoxide production, leading to an increased response to antigens.<sup>118</sup> The G-CSF protein is encoded with a single gene, in humans located on chromosome 17q11-22.<sup>119</sup> G-CSF knockout mice have reduced numbers of granulocytes, macrophages and their precursors, leading to an inability to respond to bacterial infections.<sup>120</sup> The targeted disruption of the G-CSF-receptor (G-CSF-R) in mice leads to decreased numbers of phenotypically normal circulating neutrophils with decreased hematopoietic progenitor cell numbers in the bone marrow.<sup>121</sup>

Initially, in order to produce 5 µg of pure human G-CSF, it was necessary to purify 40 liters of medium from the human bladder carcinoma cell line 5637; this work was performed under the supervision of Malcolm Moore at Memorial Sloan-Kettering Cancer Center in New York.<sup>113,114</sup> The limited amounts of G-CSF that could thus be produced precluded extensive *in vivo* experiments. In cooperation with Amgen in California, the amino acid sequence of G-CSF was deduced, making it possible to clone the cDNA for G-CSF and express it in *E. coli*, leading to the production of recombinant human G-CSF in 1986.<sup>122,123</sup> In Japan, the cDNA for murine G-CSF was isolated and characterized, showing a 72.8% homology in the amino acid sequence compared to human G-CSF.<sup>124</sup> Preclinical studies with recombinant human G-CSF were initiated and, in nonhuman primates, it was shown that G-CSF at a dose of 10 µg/kg/day increases the neutrophil count to approximately  $50 \times 10^9/\text{L}$ , with the neutrophils functioning normally *in vitro*.<sup>125</sup> In autologous bone marrow transplantation, G-CSF was shown to shorten the period of neutropenia after myeloablative chemotherapy or total body irradiation.<sup>126</sup>

In 1988, Gabrilove et al. published the first study on G-CSF in humans, describing its use in patients receiving intensive chemotherapy for transitional cell carcinoma of the urothelium.<sup>127</sup> In these patients, G-CSF increased the neutrophil counts and reduced the number of days of neutropenia, resulting in fewer infections and more patients receiving planned chemotherapy. Similar effects of G-CSF were subsequently observed in the treatment of other malignancies.<sup>128,129</sup> Patients with congenital neutropenia also benefited from the administration of G-CSF.<sup>130</sup> In over 90% of the patients with severe chronic neutropenia, the administration of G-CSF induced an increase of neutrophils  $>1.0 \times 10^9/\text{L}$ , resulting in a reduction of bacterial infections and a diminished use of antibiotics.<sup>131</sup>

When the first clinical trials of G-CSF in cancer patients were performed, it was observed that the frequency of hematopoietic colony-forming cells in the peripheral blood of these patients increased over 100-fold.<sup>132</sup> A similar effect on peripheral

blood HSPC was seen after the administration of GM-CSF in cancer patients.<sup>133</sup> This discovery paved the way for the use of mobilized peripheral blood HSPC for transplantation in humans, since it had already been shown that transplanted circulating blood cells could restore hematopoietic function in lethally irradiated animals.<sup>134–137</sup> In 1992, Sheridan et al. showed that patients receiving G-CSF-mobilized peripheral blood progenitors after high-dose chemotherapy had significantly faster hematopoietic reconstitution.<sup>138</sup> After the introduction of G-CSF as a mobilizing agent into clinical practice in the early nineties, the use of mobilized HSPC has largely replaced bone marrow as a source of stem cells for both autologous and allogeneic cell transplantation.<sup>109</sup>

### INTERLEUKIN-8 (IL-8)

Interleukin-8 (IL-8, CXCL8) is part of the CXC family of chemokines, which are known to mainly attract and activate neutrophils.<sup>139</sup> In mice, macrophage inflammatory protein 2 (MIP-2) and the murine keratinocyte-derived protein chemokine KC are the functional homologs of IL-8. Interleukin-8 is produced by monocytes, neutrophils and a variety of non-hematopoietic cells and induces the release of neutrophil proteases, such as matrix metalloproteinase-9 (MMP-9) and neutrophil elastase.<sup>139</sup> The IL-8 receptors CXCR1 and CXCR2 are located on the cell surface of neutrophils. Despite the lack of a gene coding for IL-8, murine neutrophils express a homologous receptor, termed IL-8 receptor homolog, that is able to respond to human IL-8 in addition to MIP-2 and KC.<sup>140</sup> When administered locally, IL-8 leads to an inflammatory response characterized by neutrophil accumulation and plasma protein extravasation.<sup>141</sup> Interleukin-8 can be induced by the chemokine interleukin-1 (IL-1), which was shown to rapidly mobilize HSPC in mice.<sup>142</sup> Parenteral injection of a single dose of IL-8 in mice, rats and nonhuman primates results in an immediate (within 5 minutes) phase of neutropenia, followed by a phase of neutrophilia that lasts up to several hours.<sup>143–145</sup> Between 15 and 30 minutes after IL-8 injection, coinciding with the neutropenic phase, the number of peripheral blood CFU-GM increases up to 20-fold.<sup>144</sup> Mononuclear cells that are harvested in that time period have a radioprotective capacity and long-term multilineage repopulating ability, demonstrating the capability of IL-8 to mobilize HSPC.<sup>144</sup>

### FLT3-LIGAND (FL)

Flt3-ligand is a type 1 transmembrane protein that exists in both a membrane-bound and a soluble form. It is expressed by hematopoietic and non-hematopoietic tissues and is involved in cell survival, proliferation and differentiation during early hematopoiesis.<sup>146</sup> FL binds to the tyrosine kinase type III receptor Flt3/Flk2 (CD135), which is expressed by HPC.<sup>147</sup> In steady state hematopoiesis, FL is important for lymphocyte development, but not for differentiation into myeloid lineages.<sup>148</sup> Alone

or in combination with other growth factors, FL induces the proliferation of human and murine HPC *in vitro*; *in vivo*, it induces the expansion and mobilization of HSPC in experimental animal models and humans.<sup>146,149–153</sup>

Mice and non-human primates treated with recombinant FL for 3 to 10 days have increased numbers of colony-forming units in the bone marrow, spleen and peripheral blood, with the highest frequency after 10 days of administration, showing that FL is a relatively slow mobilizing agent compared to G-CSF and IL-8.<sup>146,153</sup> In addition, peripheral blood mononuclear cells obtained from mice treated with FL for 10 days have the capacity to rescue and reconstitute multiple hematopoietic lineages following transplantation in irradiated recipient animals.<sup>146</sup> When administered in combination with G-CSF, GM-CSF or plerixafor (a CXCR4 antagonist), the addition of FL leads to the mobilization of a significantly higher number of cells with long-term, multilineage reconstitution potential.<sup>153–155</sup>

## MECHANISMS OF HSPC MOBILIZATION

After the initial reports of cytokine-induced HSPC mobilization in humans, experiments in mice have identified a number of pathways that are involved in this process. Several mechanisms have been proposed to explain how G-CSF and other cytokines affect the bone marrow and promote HSPC migration to the peripheral blood. At the start of the research described in this thesis, most studies discussed the role of neutrophils and their proteases. This role will be discussed in this chapter. Other and more recently discovered mechanisms involved in HSPC mobilization that affect the HSC niche and its cellular components will be discussed in **chapter 6**.

## NEUTROPHILS AND THEIR ROLE IN HSPC MOBILIZATION

The role of neutrophils in cytokine-induced HSPC mobilization is not uniformly defined for each mobilizing cytokine. Our research group initially described the indispensable role of neutrophils in IL-8-induced HSPC mobilization.<sup>156</sup> Following the administration of the anti-Ly6C/Ly6G monoclonal antibody RB6-8C5 to deplete neutrophils, IL-8-induced HSPC mobilization was significantly reduced in mice. Moreover, the infusion of neutrophils in neutropenic mice restored IL-8-induced HSPC mobilization, illustrating the essential role of neutrophils in this process.<sup>156</sup> Neutrophils also play an essential role in HSPC mobilization with the chemokines GRO $\beta$ /CXCL2 and GRO $\beta$ T/CXCL24.<sup>157</sup> Mice that are deficient for the G-CSF-R (G-CSF-R<sup>-/-</sup>) are neutropenic and do not mobilize after stimulation with IL-8 or cyclophosphamide, suggesting that G-CSF-R<sup>+</sup> neutrophils are required.<sup>158</sup> In mice that are chimeric for wild-type and G-CSF-R<sup>-/-</sup> bone marrow cells, treatment with G-CSF leads to the mobilization of equal proportions of both G-CSF-R<sup>-/-</sup> and G-CSF-R<sup>+/+</sup> HSPC.<sup>159</sup> This result indicates that G-CSF-induced mobilization is not dependent on the G-CSF-R expression on HSPC, but rather on a normal number

of functioning neutrophils. However, the essential role of neutrophils in G-CSF-induced HSPC mobilization was challenged in a study using transgenic mice, in which the G-CSF-R is only expressed on CD68<sup>+</sup> cells of the monocytic lineage.<sup>160</sup> As G-CSF-induced HSPC mobilization in these transgenic mice is not reduced, the results suggest that G-CSF-R signals in monocytic cells are sufficient to induce HSPC mobilization.<sup>160</sup>

## THE ROLE OF INTEGRINS IN HSPC ENGRAFTMENT AND MOBILIZATION

Integrins are heterodimeric proteins that consist of a non-covalently bound  $\alpha$  - and  $\beta$ -subunit that mediate cell-cell and cell-extracellular matrix interactions. Thus far, 18  $\alpha$ -chains and 8  $\beta$ -chains have been identified, and each combination of  $\alpha$ - and  $\beta$ -subunits leads to adhesion molecules with differential ligand specificity.<sup>161</sup> The integrin subunits  $\alpha$ 4,  $\alpha$ 5,  $\alpha$ 6,  $\alpha$ 9 and  $\beta$ 1 are expressed on the surface of HSPC, while  $\alpha$ 1- $\alpha$ 3,  $\alpha$ 8,  $\alpha$ 10 and  $\alpha$ 11 have not been detected on HSPC.<sup>162-164</sup> Beta-1-integrins are involved in HSC-MSC adhesion, as indicated by co-cultures of human bone marrow-derived MSC and CD34<sup>+</sup> HSPC.<sup>162,165</sup> *In vivo*, HSC transplantation experiments have shown that various integrins, such as LFA-1 (leukocyte function-associated antigen-1,  $\alpha$ L $\beta$ 2 integrin, CD11a/CD18), VLA-4 ( $\alpha$ 4 $\beta$ 1-integrin) and VLA-5 ( $\alpha$ 5 $\beta$ 1-integrin) are involved in the engraftment of HSC in mice and humans.<sup>166,167</sup> Besides their role in HSC engraftment, integrins are also involved in HSPC retention and mobilization from the bone marrow.<sup>168-171</sup> Our group showed that after a single injection of neutralizing anti-LFA-1 antibodies, IL-8 induced mobilization of HSPC in mice is blocked.<sup>172</sup> Moreover, injection of neutralizing antibodies to the LFA-1 ligand intercellular adhesion molecule-1 (ICAM-1) significantly inhibited IL-8 induced HSPC mobilization.<sup>172</sup> As LFA-1 is expressed on the surface of neutrophils, but not on HSC, this indicates a role for neutrophils in IL-8-induced HSPC mobilization.<sup>173</sup> The administration of antibodies against the  $\alpha$ -chain of LFA-1 enhances G-CSF-induced HSPC mobilization more than twofold as compared to the administration of G-CSF alone. In contrast, the administration of anti-LFA-1 antibodies alone does not result in HSPC mobilization.<sup>174</sup> Since G-CSF-induced mobilization is not enhanced in LFA-1 knock-out mice, this indicates that LFA-1 expressing cells, including neutrophils, mediate this synergistic effect.<sup>174</sup>

In mice, the conditional deletion of either VLA-4 or its receptor VCAM-1, which is constitutively expressed by bone marrow stromal cells, induces significant migration of HSPC towards the peripheral blood.<sup>170,171</sup> In mice and primates, blocking of the receptor-ligand interaction by either neutralizing anti-VLA-4 or anti-VCAM-1 monoclonal antibodies also results in significant HSPC mobilization.<sup>175-178</sup> Natalizumab is a recombinant humanized monoclonal antibody against the  $\alpha$ 4 subunit of VLA-4 and approved for treating patients with multiple sclerosis and Crohn's disease. Treatment with natalizumab results in the increase of peripheral blood CD34<sup>+</sup> cells

in these patients.<sup>179-181</sup> The concept of blocking the VLA-4-VCAM-1 interaction to induce HSPC mobilization is currently under investigation for use in humans.<sup>182</sup>

## PROTEASES IN CYTOKINE-INDUCED HSPC MOBILIZATION

Neutrophils become activated by G-CSF, as is demonstrated by an increased expression of Fc receptor I (FcγRI, CD64), CD11b, CD66b and FcRIII (CD16) following G-CSF administration.<sup>183,184</sup> Upon the administration of G-CSF, proteases including neutrophil elastase (NE) and cathepsin G (CG) are released from the granules of activated neutrophils. These proteases accumulate in the bone marrow during HSPC mobilization, leading to a highly proteolytic environment.<sup>185</sup> Subsequently, VCAM-1 is cleaved by NE and CG, causing an increase in soluble VCAM-1 in the peripheral blood.<sup>186</sup> Moreover, NE, CG and MMP-9 cleave the interaction between CXCL12 and its receptor CXCR4, expressed by HSPC and mature leukocytes, leading to the egress of HSPC from the bone marrow to the peripheral blood.<sup>187,188</sup> Earlier studies from our group had already suggested a role for MMP-9 in IL-8-induced HSPC mobilization, as function-blocking anti-MMP-9 antibodies inhibit IL-8-induced mobilization in non-human primates.<sup>189</sup>

Proteolytic activity also affects the interaction between SCF and its receptor c-Kit (CD117).<sup>190</sup> Both human and mouse c-Kit are cleaved by NE, CG and MMP-9, leading to reduced c-Kit expression on mobilized human and murine HSPC.<sup>190</sup> Cleavage of SCF by MMP-9 has been reported during treatment with the chemotherapeutic agent 5-fluorouracil.<sup>191</sup>

Similar to mice, the levels of MMP-9 and NE in humans increase after 3 to 5 days of G-CSF administration; these levels correlate with the extent of CD34<sup>+</sup> mobilization.<sup>192</sup> Nevertheless, the notion that proteases are essential for HSPC mobilization has been challenged by experiments in transgenic mice that are deficient for one or more proteases. The targeted deletion of MMP-9, NE and CG in C57BL/6 mice did not affect the mobilizing capacity of HSPC.<sup>193</sup> This result can be explained by the existence of redundant pathways in these mice. Furthermore, in response to G-CSF, the C57BL/6 mouse strain, which is known for having a relatively decreased mobilizing capacity, has a constitutively lower level of active neutrophil proteases in the bone marrow than strains that mobilize well, such as BALB/c or DBA/2; this result correlates with a higher serum level of alpha-1 antitrypsin (AAT), the main inhibitor of NE.<sup>194</sup> Therefore, the physiologic significance of proteases in stem cell mobilization remains uncertain.<sup>195</sup>

## PROTEASE INHIBITORS IN THE HEMATOPOIETIC STEM CELL NICHE

Under physiologic conditions, the accumulation of high levels of proteolytically active proteases is strictly regulated by the presence of protease inhibitors.<sup>196,197</sup> Protease inhibitors that can inhibit neutrophil-derived serine proteases are called

serpins, of which *serpina1* (or alpha-1-antitrypsin (AAT)) and *serpina3* (or alpha-1-antichymotrypsin (ACT)) inhibit NE and CG respectively.<sup>196</sup> Both AAT and ACT are observed in bone marrow extracellular fluids.<sup>198</sup> AAT is locally produced, mainly by osteoblasts, suggesting a protective role for this protease inhibitor in the HSC niche.<sup>199</sup> Matrix metalloproteinases are inhibited by so-called tissue inhibitors of metalloproteinases (TIMPs).<sup>200</sup> Although MMPs play a role in HSPC mobilization, the role of TIMPs in HSPC mobilization is probably less critical.<sup>157,189,201</sup> Protease inhibitor expression is tightly regulated in the bone marrow. Upon G-CSF or cyclophosphamide administration, levels of AAT and ACT decrease significantly in the bone marrow, with a concomitant increase in NE and CG activity.<sup>198</sup> This subsequently leads to the cleavage of adhesion molecules on the surface of HSPC in the bone marrow. Our group showed that low-dose total body irradiation (0.5 Gy) of mice significantly inhibited G-CSF and IL-8-induced HSPC mobilization by increasing the levels of bone marrow AAT.<sup>202</sup> Furthermore, the administration of human AAT almost completely blocked IL-8-induced HSPC mobilization, demonstrating an important role for protease inhibition in HSPC retention in the bone marrow.<sup>202</sup> These experiments indicate that maintaining a balance between proteases and their inhibitors is an essential component in the regulation of homeostasis within the bone marrow microenvironment and the mobilization of HSPC.<sup>201-203</sup>

# SCOPE OF THIS THESIS

Understanding the mechanisms of HSPC mobilization is fundamental to develop novel strategies and to optimize current HSPC mobilization protocols. This thesis focuses on the mechanisms that underlie HSPC mobilization, using both mouse models and observations in humans.

In **chapter 2**, we investigate the kinetics of HSC and HPC mobilization following the administration of Flt3-ligand in a murine model for stem cell mobilization. Because of the slow mobilizing capacity of this cytokine, a time frame was provided that allowed us to detect differences in the mobilized hematopoietic cell populations at different time points in the mobilization process.

The administration of mobilizing cytokines results in the egress of HSC and HPC from the bone marrow towards the peripheral blood. Hypothetically, repeated cytokine administration could lead to the depletion of the bone marrow HSPC pool with a subsequent loss of mobilizing capacity. In **chapter 3**, we describe the effects of repeated *in vivo* administration of recombinant human G-CSF and murine recombinant G-CSF for up to 12 cycles/24 weeks in a murine model.

G-CSF-induced HSPC mobilization is associated with the release of neutrophil-derived proteases. We have previously shown that AAT inhibits these proteases in mice, resulting in decreased HSPC mobilization. However, the role of protease inhibition during HSPC mobilization in humans is unclear. In **chapter 4**, we describe the relationship between AAT and HSPC in steady state and during G-CSF-induced mobilization in humans.

Modulation of the HSC niche plays an essential role in cytokine-induced HSPC mobilization. Hypothetically, *in vivo* administration of mesenchymal stromal cells (MSC), an essential component of the HSC niche, could initiate changes in the niche that affect subsequent HSC mobilization. In **chapter 5** we explore the effects of MSC on the HSC niche in a murine model of G-CSF-induced HSPC mobilization.

Finally, in **chapter 6** we provide a review of the events that occur during cytokine-induced HSPC mobilization. An extensive overview is given on the changes in the HSC niche during cytokine-induced mobilization. In addition, we review the use of G-CSF and other mobilizing agents in current and novel strategies to mobilize bone marrow-derived HSPC.

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# CHAPTER 2

## HEMATOPOIETIC STEM –AND PROGENITOR CELLS ARE DIFFERENTIALLY MOBILIZED DEPENDENT ON THE DURATION OF FLT3-LIGAND ADMINISTRATION

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Published in: Haematologica. 2010; 95(7): 1061-67

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# ABSTRACT

## BACKGROUND

Flt3-ligand (FL) is a cytokine that induces relatively slow mobilization of hematopoietic cells in animals and humans *in vivo*. This provides a time-frame to study hematopoietic stem and progenitor cell (HSC/HPC) migration kinetics in detail.

## DESIGN AND METHODS

Mice were injected with FL (10 µg/day, intraperitoneally) for 3, 5, 7 and 10 days. Mobilization of HSC/HPC was studied using colony-forming-unit granulocyte/monocyte (CFU-GM) and cobblestone-area-forming-cell (CAFC) assays. The radioprotective capacity of mobilized peripheral blood mononuclear cells (PBMC) was studied by transplantation of  $1.5 \times 10^6$  FL-mobilized PBMC into lethally irradiated (9.5 Gy) recipients.

## RESULTS

HPC mobilization was detected from day 3 onwards and prolonged administration of FL showed a steady increase in mobilized progenitor cells. Administration of FL for 10 days lead to a 5.5-fold increase in CAFC-week 4 and a 5.0-fold increase in CAFC-week 5, compared to FL administration for 5 days. Furthermore, transplantation of 5-day FL-mobilized PBMC did not radioprotect lethally irradiated recipients, whereas 10-day FL-mobilized PBMC radioprotected 100% of the recipients with significant multilineage donor chimerism.

Co-administration of IL-8 and FL led to a synergistic enhancement of HSC/HPC mobilization on day 3 and 5, compared to FL or IL-8 alone.

## CONCLUSIONS.

These results indicate that HPC and HSC show different mobilization kinetics in response to FL, resulting in preferential mobilization of HPC at day 5, followed by HSC mobilization at day 10.

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## INTRODUCTION

Hematopoietic stem cells (HSC) are capable of extensive self renewal and confer long-term multilineage repopulating ability upon transfer into myeloablated recipients. Under steady state conditions, HSC comprise a small proportion (0.01%) of bone marrow cells, where they serve as a reservoir of mainly quiescent cells that have the ability to be recruited into cycle upon hematopoietic stress.<sup>1</sup> The immediate progeny of hematopoietic stem cells are hematopoietic progenitor cells (HPC), which retain full lineage potential but have limited self-renewal capacity.<sup>2</sup> They are relatively more abundant than HSC and able to transiently reconstitute myeloablated recipients. The absolute frequency of HPC/HSC in the peripheral blood is under normal circumstances relatively low, but is considerably increased by mobilizing HPC and HSC from the bone marrow using a variety of chemokines and cytokines.<sup>3,4</sup>

The tyrosine kinase type III receptor *flt3/flk2* (fms-like tyrosine kinase receptor-3/fetal liver kinase-2; CD135), is primarily expressed by hematopoietic progenitor cells, as well as multipotent progenitor cells.<sup>5</sup> Its ligand, *Flt3 ligand* (FL), is a type 1 transmembrane protein that exists in both a membrane bound and a soluble form. FL is a cytokine which is involved in cell survival, proliferation, and differentiation during early hematopoiesis.<sup>6</sup> In addition, FL is important for lymphocyte (B cell and T cell) development, but not for differentiation into myeloid lineages.<sup>7-9</sup> In contrast to the more limited expression of *flt3* on hematopoietic cells, FL mRNA has been found in various hematopoietic and non-hematopoietic tissues.<sup>10</sup>

Alone, or in combination with other growth factors, FL stimulates the proliferation of highly enriched human and murine HSC *in vitro* and leads to expansion and mobilization of progenitor cells in animals and humans *in vivo*.<sup>6,11-15</sup> Mice and non-human primates treated with recombinant FL for 3 to 10 days have increased numbers of colony-forming units in bone marrow, spleen and peripheral blood, with the highest frequency after 10 days of administration, showing that FL is a relatively slow mobilizing agent.<sup>6,15-17</sup> In addition, peripheral blood mononuclear cells (PBMC) obtained from mice treated with FL for 10 days also have the capacity to rescue and reconstitute multiple hematopoietic lineages following transplantation in irradiated recipient animals.<sup>6</sup> When administered in combination with G-CSF or GM-CSF, administration of FL leads to mobilization of significantly higher numbers of cells with long-term, multilineage reconstitution potential.<sup>15</sup>

Previously, we have shown that IL-8 significantly increases HPC/HSC mobilization in combination with several other hematopoietic growth factors like G-CSF, GM-CSF, IL-3 and SCF<sup>18</sup>. However, IL-8 has not been studied in combination with FL. Since FL is a slow mobilizing agent, the subsequent addition of a very quick mobilizing agent such as IL-8 (i.e. optimal HSC/HPC mobilization at 20 minutes after injection) might accelerate HPC/HSC mobilization.

In the current study, we analyzed the kinetics of FL-induced HSC/HPC mobilization. Three to 5 days of FL administration leads to detectable numbers of mobilized HPC, whereas administration of FL for 10 days results in mobilization of HSC with long-term repopulating ability. These results indicate that HPC and HSC show different mobilization kinetics in response to FL. In addition, combined administration of FL and IL-8 synergistically enhanced the recruitment of HSC/HPC.

## DESIGN AND METHODS

### ANIMALS

Male Balb/c and C57BL/6 mice (8-12 weeks old) were purchased from Charles River (Maastricht, The Netherlands). C57BL/6-Ly5.1 congenic recipients were bred from our own facilities and were used at 6-12 weeks of age. The animals were fed commercial rodent chow and acidified water and were maintained in the animal facility of the Leiden University Medical Center under conventional conditions. Bone marrow cell recipients received sterilized regular chow for a period of 4 weeks directly after bone marrow transplantation. In addition, from week -1 until +4 after bone marrow cell transplantation, drinking water was supplied with antibiotics (polymixin B (Bupha Uitgeest, The Netherlands), ciprofloxacin (Bayer b.v., Mijdrecht, The Netherlands), amphotericin B (Bristol-Myers Squibb, Woerden, The Netherlands) and saccharose. All experimental protocols were approved by the institutional ethics committee on animal experiments.

### GROWTH FACTORS

All growth factors were administered as an intraperitoneal (i.p.) injection in 0.2 ml 0.1% Bovine Serum Albumin/Phosphate Buffered Saline (0.1% BSA/PBS). Recombinant human Flt3-ligand (FL; kindly provided by Amgen, Thousand Oaks, California, USA) and recombinant-human-G-CSF (filgrastim, Amgen) were administered at 10 µg i.p. per day. IL-8 (Novartis, Vienna, Austria) was administered as a single, 30 µg i.p. injection. Control mice were injected with 0.2 ml 0.1% BSA/PBS alone (referred to as PBS).

### PREPARATION OF CELL SUSPENSIONS

Twenty-four hours after the last FL or G-CSF injection or 20 minutes after IL-8 administration, mice were sacrificed by CO<sub>2</sub> asphyxiation and peripheral blood and bone marrow cells were harvested. Under sterile conditions, peripheral blood-derived mononuclear cell suspensions (PBMC) were obtained by Ficoll (LUMC Pharmacy, Leiden, The Netherlands) density separation. Bone marrow cells were harvested by flushing a femur under sterile conditions with RPMI 1640, containing

500 µg/ml penicillin, 250 µg/ml streptomycin, 10% heparin and 2% fetal bovine serum (FBS, Gibco, Grand Island, NY). Spleen cells were obtained by straining the spleen through a 100 µm pore filter (BD Falcon, Franklin Lakes, New Jersey, USA) and washing the cell suspension twice in a RPMI 1640 solution containing penicillin, streptomycin and FBS, as described above.

### MONOCLONAL ANTIBODIES

For lineage analysis, cells were stained with FITC-labeled anti-GR-1 (clone RB6-8C5) and anti-CD90.2 (Thy-1.2, clone 53-2.1) and PE-labeled anti-B220 (clone RA3-6B2), anti-CD11b (clone M1/70) and anti-CD3 (clone 145-2C11). To detect chimerism in transplanted recipient mice, cells were stained with PE-labeled anti-CD45.1 (clone A20) and/or FITC-labeled anti-CD45.2 (clone 104). All monoclonal antibodies used in this study were obtained from Pharmingen (San Diego, CA).

### PROGENITOR CELL ASSAY

The Colony Forming Units - Granulocyte Macrophage (CFU-GM)-assay is specifically designed to quantify the frequency of HPC. CFU-GM were cultured by resuspending  $5 \times 10^5$  peripheral blood cells,  $5 \times 10^4$  bone marrow cells or  $1 \times 10^6$  spleen cells in a 3.5 cm<sup>2</sup> Petri dish containing 1 ml of a 40% methylcellulose, 30% IMDM and 20% FBS solution with the addition of 1.25 ng/ml recombinant murine Granulocyte-Macrophage-Colony-Stimulating-Factor (GM-CSF). After 7 days of incubation at a temperature of 37 °C in a 5% CO<sub>2</sub> environment, colonies (defined as a cluster of 20 or more cells) were scored using an inverse light microscope.

### COBBLESTONE AREA FORMING CELL ASSAY

The cobblestone area forming cell (CAFC) assay measures a spectrum of hematopoietic cells and allows early progenitor cells (arising at day 7-14 of culture) to be separated from more primitive HSC with long-term repopulating ability (day 28-35 CAFC). Confluent stromal layers of FBMD-1 cells in flat-bottom 96-well plates (Falcon, Etten-Leur, The Netherlands) were overlaid with various dilutions of PBMC to allow limiting dilution analysis of the precursor cells forming hematopoietic clones under the stromal layers. To assay a particular cell suspension, we used 8 dilution steps differing with a factor of 2.5, with 15 wells per dilution. The cells were cultured at 33 °C, 7% CO<sub>2</sub> and were fed weekly by changing half of the medium. Between 7 and 42 days after overlay, all wells were inspected at weekly intervals and scored positive if at least one phase-dark hematopoietic clone (cobblestone area, at least 5 cells) was observed. The CAFC frequencies were calculated using Poisson statistics.<sup>19</sup>

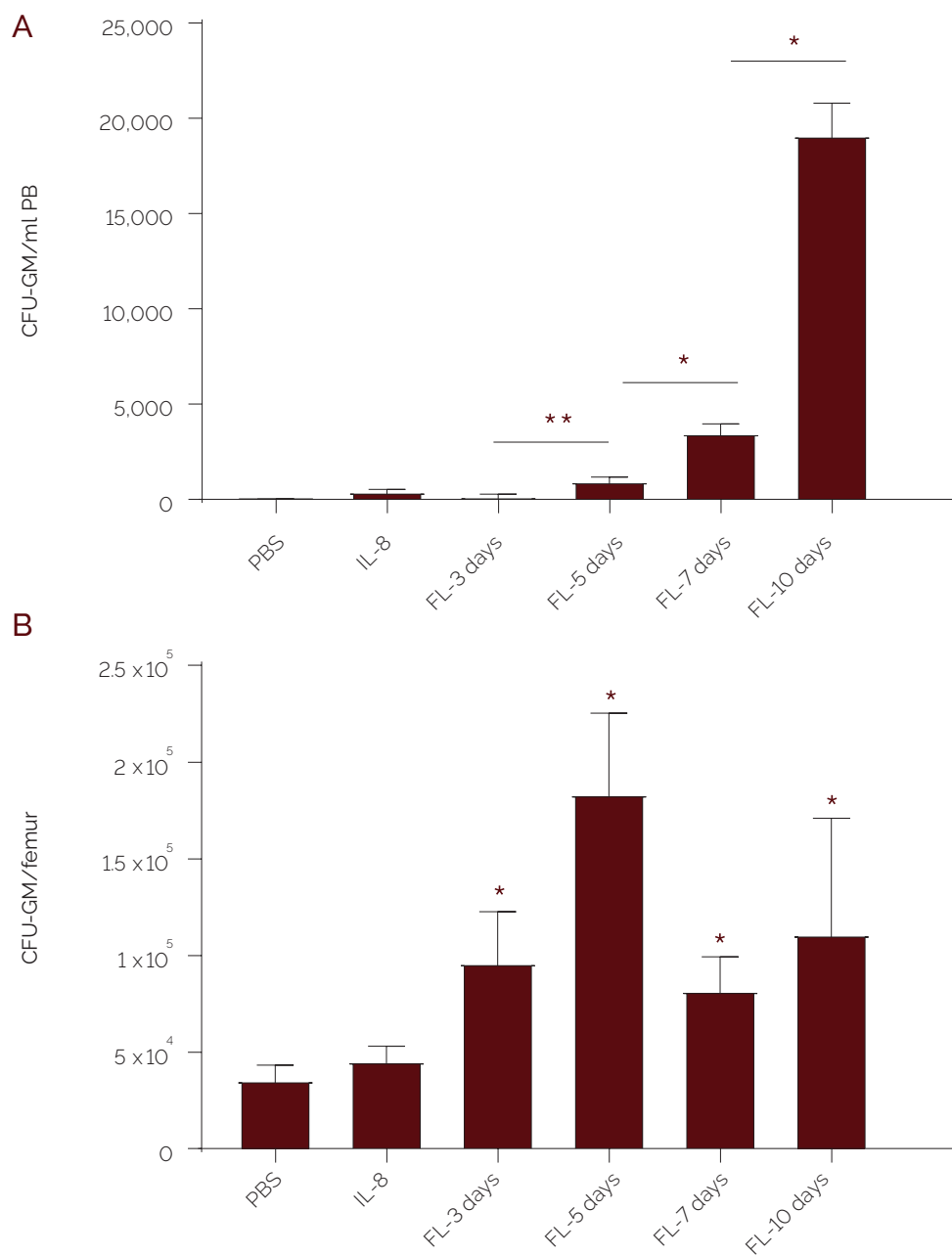


**FIGURE 1. Flt3-ligand administration induced HPC mobilization.**

FL was administered for 3, 5, 7 or 10 days. (A) Colony-forming cells as determined by CFU-GM assay in peripheral blood (n= 10-20 per group; results are shown as mean  $\pm$  SEM; \*  $p < 0.0001$ , \*\*  $p = 0.0007$ ). (B) Colony-forming cells in bone marrow expressed as CFU-GM/femur (n= 10-20 per group; results are shown as mean  $\pm$  SEM; \*  $p < 0.02$  compared to PBS).

## PERIPHERAL BLOOD MONONUCLEAR CELL TRANSPLANTATION

Prior to peripheral blood mononuclear cell transplantation (PBMC) transplantation, recipients were irradiated in perspex chambers with a linear accelerator (Philips SL 75-5/6 mV, Philips Medical Systems, Best, The Netherlands) at a dose rate of 98 cGy/min. Total doses of 9.5 Gy were administered. Four to 6 hours following total body irradiation, PBMC were injected via caudal vein injection in 0.2 ml of saline, containing 0.2% bovine serum albumin.

## STATISTICAL ANALYSIS

Statistical differences were determined using the Student t-test and the log rank test using GraphPadPRISM (GraphPad Software, San Diego, CA). P values of <0.05 were considered statistically significant.

# RESULTS

## PROLONGED ADMINISTRATION OF FLT3-LIGAND LEADS TO INCREASED MOBILIZATION OF HPC

To determine the mobilizing capacity of FL, Balb/c mice were given 10 µg FL i.p. as a daily dose during 3, 5, 7 or 10 days. Twenty-four hours after the last FL injection, peripheral blood was harvested and the CFU-GM colony formation was determined. On day 3 of FL administration, HSC/HPC mobilization was 9.5-fold increased compared to PBS-treated controls. HSC/HPC migration was even further increased on day 5, 7 and 10 following FL administration compared to PBS administration (50-fold, 153-fold and 823-fold respectively for 5, 7 and 10 days;  $p < 0.001$  for comparison between all groups; **Figure 1A**).

Bone marrow CFU-GM was significantly increased after FL administration as compared to PBS (CFU-GM/femur  $\pm$  SEM: PBS 35,839  $\pm$  7,433, FL 3 days 96,532  $\pm$  26,140, 5 days 183,819  $\pm$  41,515, 7 days 82,210  $\pm$  16,963 and 10 days 111,504  $\pm$  59,750,  $p < 0.02$  for comparison between PBS and 3, 5, 7 or 10 days FL, **Figure 1B**). The highest number of BM CFU-GM was seen at day 5 of FL administration, although no significant difference was found between the FL treated groups (FL 3 days vs. FL 5 days:  $p = 0.12$ , FL 5 days vs. FL 7 days:  $p = 0.10$ , FL 7 days vs. FL 10 days:  $p = 0.52$ ).

## MIGRATION OF LONG-TERM REPOPULATING STEM CELLS IS DEPENDENT ON THE DURATION OF FLT3-LIGAND ADMINISTRATION

In order to determine the frequency of primitive HSC in the peripheral blood of FL-mobilized mice, CAFC frequency was determined. PBMC obtained from mice that received FL for 5 or 10 days were compared to those from PBS-injected controls. Similar to the number of CFU-GM, the highest proportion of CAFC-day

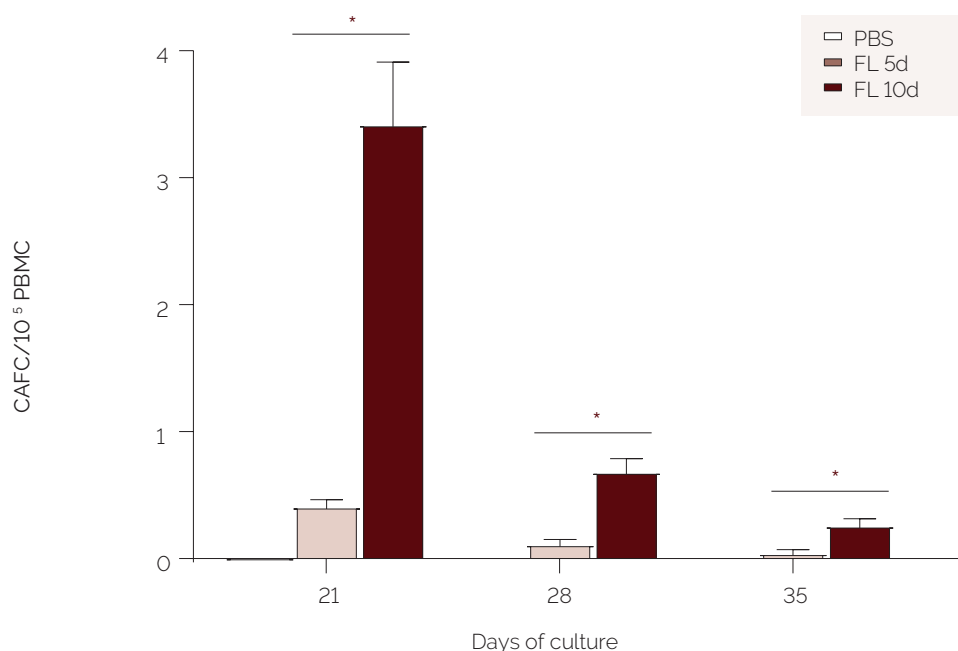


**FIGURE 2. Mobilization of long-term repopulating stem cells is dependent on the duration of FL administration.**

PBMC of 5 or 10 day FL mobilized mice ( $n=12$  per group) were analyzed for CAFC frequency. Results are shown of two independent experiments as mean  $\pm$  SEM, \*  $p<0.001$ .

7 was found following 10 days of FL-administration (data not shown). Upon the duration of the culturing, the CAFC-frequency in both groups steadily decreased. After 28-35 days of culture, the frequency of CAFC was significantly higher in the group that received FL for 10 days as compared to the group that received FL for 5 days (CAFC-day 28  $\pm$  SEM: FL 5 days  $0.16 \pm 0.02$ , FL 10 days  $0.69 \pm 0.10$  per  $10^5$  PBMC; CAFC-day 35  $\pm$  SEM: FL 5 days  $0.096 \pm 0.017$  and FL 10 days  $0.27 \pm 0.04$  per  $10^5$  PBMC;  $p<0.001$  for comparison between FL 5 days and 10 days at 28 days and 35 days; **Figure 2**). In PBS-injected controls, the frequency of HSC was below the detection limit of the CAFC-assay.

These results indicate that the frequency of primitive HSC in the peripheral blood is dependent on the duration of FL administration and is significantly enhanced after 10 days of FL administration as compared to 5 days of FL administration.

### PBMC FROM 10 DAY FLT3-LIGAND MOBILIZED MICE ARE RADIOPROTECTIVE *IN VIVO*

To assess the long term repopulating ability of FL-mobilized HSC/HPC, radioprotection-assays were performed. To this end,  $1.5 \times 10^6$  PBMC obtained from Balb/c mice that received daily injections of FL for 5 or 10 days, were transplanted

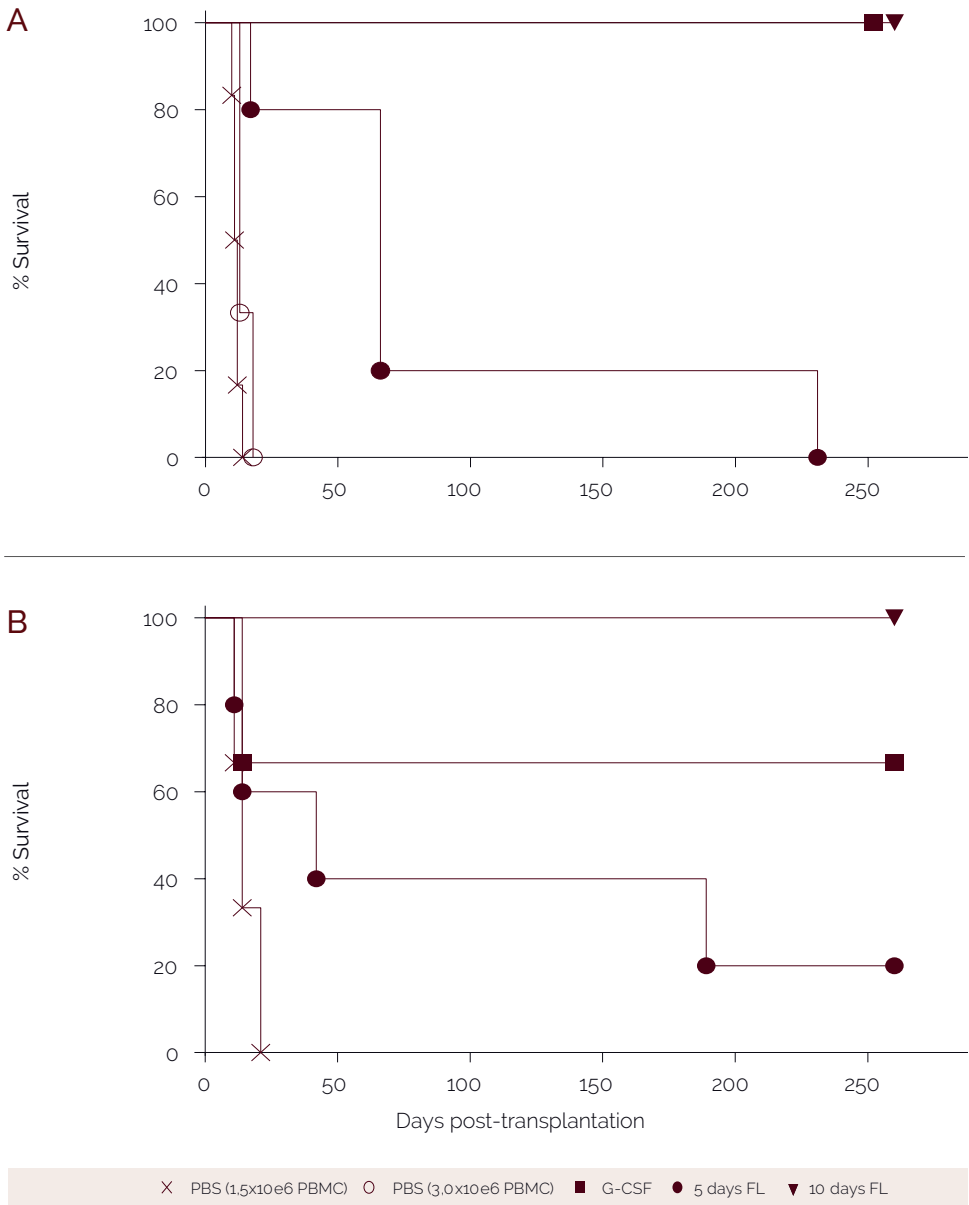


**FIGURE 3** PBMC from 10 day FL mobilized mice are radioprotective *in vivo*.

**(A)** Lethally irradiated recipient Balb/c mice (9.5 Gy;  $n=5$  per group) were transplanted with  $1.5 \times 10^6$  PBMC obtained from Balb/c donors treated with 5 or 10 days of FL or 4 days of G-CSF. Control mice received  $1.5 \times 10^6$  or  $3.0 \times 10^6$  PBMC obtained from PBS-injected donors. **(B)** Lethally irradiated C57Bl/6 Ly5.1 recipients (9.5 Gy;  $n=5$  per group) received  $1.5 \times 10^6$  PBMC obtained from C57Bl/6 Ly5.2 donors treated with 5 or 10 days of FL or 4 days of G-CSF. As a control,  $1.5 \times 10^6$  PBMC obtained from PBS-injected donors were administered.

into lethally irradiated (9.5 Gy) syngeneic recipient mice. Controls received either  $1.5 \times 10^6$  or  $3.0 \times 10^6$  PBMC obtained from PBS-injected donors or  $1.5 \times 10^6$  PBMC obtained from donors that were injected with G-CSF for 4 days.

The mean survival time (MST) for recipients of  $1.5 \times 10^6$  PBS-injected donor cells was 11.5 days and for recipients of  $3.0 \times 10^6$  PBMC obtained from PBS-injected donors 13 days. PBMC that were harvested following FL administration for 5 days were not radioprotective *in vivo*. Although their MST exceeded that of the PBS control group (66 days vs. 11.5 days,  $p=0.001$ ), all mice had died within 231 days. This was in sharp contrast with the PBMC that were harvested following FL administration for 10 days, showing 100% radioprotection (MST > 260 days, **Figure 3A**). Similarly, transplantation of  $1.5 \times 10^6$  PBMC obtained from G-CSF-mobilized donors showed a radioprotection rate of 100% (MST > 260 days).

At several time-points following PBMC transplantation, multi-lineage reconstitution analysis of peripheral blood cells was performed during and at the end of the experiment at 260 days. In all FL-10 days and G-CSF recipient mice granulocyte, monocyte, T-lymphocyte and B-lymphocyte lineages were detected (data not shown).

To specifically determine donor chimerism, a second set of transplantation experiments was performed. In this experiment lethally irradiated (9.5 Gy) C57BL/6 Ly5.1 recipients received  $1.5 \times 10^6$  PBMC, obtained from C57BL/6 Ly5.2 donors that received daily injections of FL for 5 or 10 days or G-CSF for 4 days as a control. The mean survival time (MST) for recipients of PBS-injected donor cells was 14 days and 1 out of 3 recipients of PBMC obtained from G-CSF-mobilized donors died at day 14. At 27 weeks after transplantation, 1/5 recipients in the FL-5 days group (MST 42 days) and 5/5 recipients in the FL-10 day group were alive (**Figure 3B**). Long term donor chimerism was determined at week 27 post transplantation by FACS analysis. In the FL-10-days and the G-CSF group donor chimerism was  $90.9\% \pm 1.3\%$  and  $91.5\% \pm 0.51\%$  respectively. However, in the FL-5-days group, significant donor chimerism (i.e. 84.0%) was seen in only 1 of the 2 surviving recipients. Long term multilineage donor chimerism showed an identical pattern in all lineages that were tested (granulocytes, lymphocytes and monocytes), with significant donor chimerism in all mice in the FL-10 days and G-CSF-group (**Table 1**).

Together, these results indicate that FL-induced mobilization for 5 days preferentially mobilizes HPC, whereas 10 days of FL administration induces the mobilization of HSC with long-term repopulating ability *in vivo*.

### **FLT3-LIGAND-INDUCED MOBILIZATION IS SYNERGISTICALLY ENHANCED BY COMBINED ADMINISTRATION OF IL-8**

Previously, it has been shown that the mobilizing capacity of FL could be synergistically enhanced by co-administration of G-CSF.<sup>15</sup> To investigate whether

**TABLE 1.** Long term donor chimerism at week 27 post-transplantation.

|               | No. alive/<br>total | Mean<br>Survival<br>Time (days) | DONOR CHIMERISM (% $\pm$ SD) |                   |                    |
|---------------|---------------------|---------------------------------|------------------------------|-------------------|--------------------|
|               |                     |                                 | Gr-1 <sup>Hi</sup>           | B220 <sup>+</sup> | CD11b <sup>+</sup> |
| FL 10<br>days | 5/5                 | > 189                           | 80.3 $\pm$ 33.4              | 99.7 $\pm$ 0.3    | 95.0 $\pm$ 2.4     |
| FL 5<br>days  | 1/5                 | 42                              | 97.9                         | 99.0              | 91.8               |
| G-CSF         | 2/3                 | > 189                           | 95.7 $\pm$ 3.2               | 99.7 $\pm$ 0.1    | 95.3 $\pm$ 1.0     |
| PBS           | 0/5                 | 14                              | -                            | -                 | -                  |

*Lethally irradiated C57BL/6 Ly5.1 recipients were transplanted with  $1.5 \times 10^6$  PBMC, obtained from C57BL/6 Ly5.2 donors that received daily injections of FL for 5 or 10 days, G-CSF for 4 days or PBS as a control. Long term donor multilineage chimerism was determined in the chimeric mice at week 27 post transplantation by FACS analysis.*

co-administration of FL and IL-8 had a synergistic effect on the mobilization of HPC and HSC, FL was administered for 3, 5 and 7 days. Twenty-four hours after the last FL-injection, 30  $\mu$ g IL-8 was administered i.p. Twenty minutes following IL-8 injection, the mice were sacrificed and the frequency of CFU-GM was determined in peripheral blood and bone marrow. Co-administration of IL-8 and FL induced a significant increase of mobilization on day 3 and 5, compared to FL or IL-8 alone (5.85-fold and 2.57-fold increase of FL + IL-8 compared to FL alone for 3 and 5 days respectively;  $p < 0.001$  and  $p = 0.003$  respectively, **Figure 4A**). In contrast, co-administration of FL for 7 days and IL-8 did not further increase the number of CFU-GM (0.95-fold increase of FL + IL-8 compared to FL alone;  $p = 0.78$ , **Figure 4A**). No difference in CFU-GM/femur was seen between the groups that received FL as a single agent or with subsequent IL-8 administration (**Figure 4B**).

Transplantation of  $1.5 \times 10^6$  PBMC obtained from 5-day FL + IL-8-mobilized donors showed a radioprotection rate of 100% (MST > 300 days). By comparison, PBMC that were harvested following a single injection of IL-8 showed a 60% long-term radioprotection rate, whereas PBMC that were harvested following FL administration for 5 days did not show long-term radioprotection *in vivo* (**Figure 4C**).

Together, these data indicate that combined administration of FL for 5 days and IL-8 induces the mobilization of radioprotective HSC.

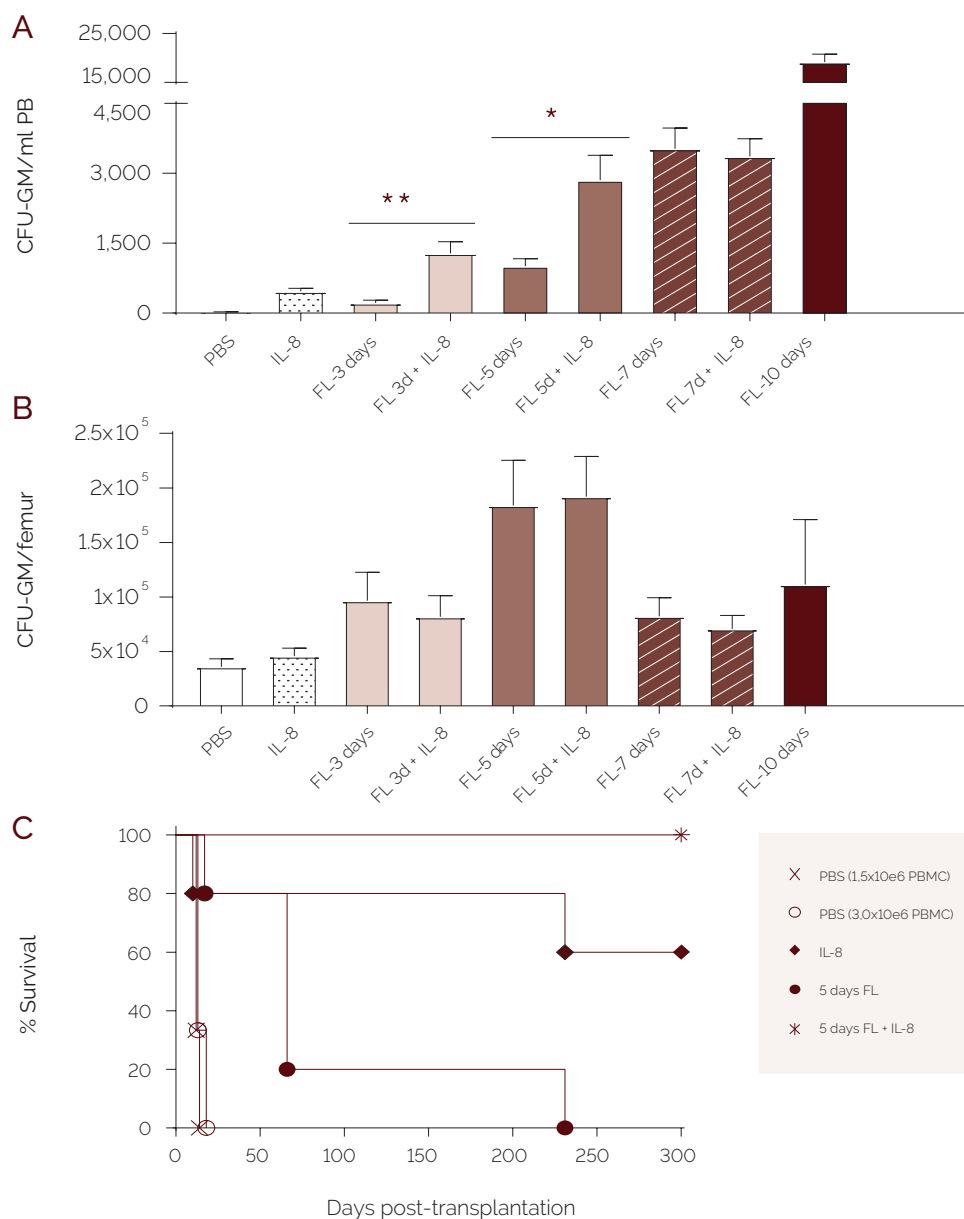


**FIGURE 4** Combined administration of FL and IL-8 synergistically enhances HPC/HSC mobilization.

Effect of co-administration of a single injection of IL-8, 24 hours after administration of FL for 3, 5 or 7 days on the number of CFU-GM in (A) peripheral blood and (B) bone marrow; results are shown as mean  $\pm$  SEM, \* $p < 0.001$ , \*\* $p < 0.05$ . (C) Lethally irradiated recipient mice (9.5 Gy;  $n = 5$ ) were transplanted with  $1.5 \times 10^6$  PBMC obtained from mice treated with 5 days of FL, a single injection of IL-8 or a combination of both. Controls received  $1.5 \times 10^6$  or  $3.0 \times 10^6$  PBMC obtained from PBS-treated donors.

## DISCUSSION

Previously, it has been shown that FL mobilizes large numbers of PBMC into the circulation of mice, with a maximum effect after 10 days of administration.<sup>6,13-15</sup> However, the qualitative content of these mobilized cells with respect to their short- and long-term repopulating ability has not been elucidated to its full extent. In the current study, we addressed this issue.

Long term (LT)-HSC, short-term (ST)-HSC and HPC show similar multilineage capabilities, but differ in their self-renewal and proliferative capacity. When LT-HSC differentiate into ST-HSC and next into HPC, their self-renewal capacity progressively declines.<sup>20</sup> Previous studies have shown that LT-HSC fail to radioprotect conditioned mice and are incapable of rapid reconstitution because of their quiescent state.<sup>21</sup> In lethally irradiated recipients, primitive ST-HSC are necessary for rapid replenishment of myeloid and erythroid progenitors, thereby providing early rescue from lethal myeloablation. However, for long-term repopulation LT-HSC are required. In accordance with others, we found that administration of FL for 10 days leads to significant mobilization of ST-HSC and LT-HSC, as was shown by their capacity to radioprotect lethally irradiated recipients.<sup>15</sup> Using donor chimerism analysis at several time points following transplantation, we showed that the survival of these mice was indeed due to the engraftment of mobilized donor HSC.

Although a significant number of HPC was mobilized in mice treated with FL for 5 days (represented as CFU-GM and CAFC-day 7 in their respective assays), the frequency of repopulating HSC (represented as CAFC-day 28 /35) in the peripheral blood was very low. Furthermore, PBMC obtained from mice that were injected with FL for 5 days did not radioprotect lethally irradiated mice. This indicates that LT-HSC were mobilized in numbers that were below the threshold to radioprotect lethally irradiated recipients. These results point towards different mobilization kinetics for HPC and HSC in response to FL, resulting in preferential mobilization of HPC at day 5, followed by an increase in HSC mobilization from day 5 to 10. A possible explanation for this finding could be that HPC are more easily recruited from their bone marrow compartment compared to LT-HSC.

It is interesting to note that LT-HSC and ST-HSC activity is correlated with Flt3-expression. In mouse bone marrow, c-Kit<sup>hi</sup>Lin<sup>neg</sup>Sca1<sup>pos</sup> (KLS) cells with absent Flt3-expression provide radioprotection and long-term reconstitution in ablated recipients, whereas purified Flt3<sup>+</sup> KLS cells fail to rescue.<sup>22</sup> This suggests that mobilized LT-HSC are not directly targeted by FL. However, since LT-HSC are indeed mobilized by FL there should be an alternative explanation for this phenomenon. One explanation could be an indirect effect of FL on LT-HSC by way of interacting with the CXCL12/CXCR4 signaling pathway.<sup>23</sup> Alternatively, neutrophils may play a role in this process, since neutrophils are increased in the bone marrow upon FL administration and are

indispensable for cytokine (IL-8 -and G-CSF)-induced HSC mobilization.<sup>6,16,17</sup>

Previously, it was shown that FL administration leads to an increase in BM-derived CFU-GM, BFU-E and CFU-GEMM, with a maximum at day 3 of FL treatment. From day 3 onwards, BM-CFU begin to decrease with a concurrent increase in splenic and PB-CFU which peaks at day 8-10 of FL treatment.<sup>6</sup> In our study we show a steady increase in BM CFU-GM after FL treatment for 3 and 5 days with a subsequent, although not significant, decrease on day 5 to 10. This suggests that CFU expansion occurs in the bone marrow prior to HPC/HSC mobilization.

The mobilizing capacity of FL was significantly enhanced when IL-8 was administered after 3 and 5 days of FL administration, with an increase in PB CFU-GM and superior long-term survival of lethally irradiated recipients. Our results are in accordance with data from studies where FL was combined with G-CSF, showing a significant increase of PB CFU-GM after combined administration of FL and G-CSF as compared to the administration of either cytokine as a single agent.<sup>6,15,24,25</sup> More specifically, in the study by Brasel et al., the highest synergy of combined FL and G-CSF administration was seen at day 6, whereas at day 10 the synergistic effect of G-CSF co-administration had disappeared, reflecting our results with co-administration of IL-8.<sup>6</sup> An explanation for the superior overall survival in the FL + IL-8 treated group could be the higher numbers of mobilized HPC in the FL + IL-8 group in comparison with IL-8 or 5 days of FL alone (as shown by the higher frequency of CFU-GM in the FL + IL-8 group in **Figure 4A**). These HPC are necessary for early survival after lethal irradiation and facilitate engraftment of LT-HSC that sustain long term survival of the recipient mice. An alternative explanation could be that FL treatment results in neutrophil expansion in the bone marrow as was shown by Brasel et al., facilitating more efficient stem cell mobilization in response to additional FL treatment or IL-8 injection.<sup>6</sup>

In summary, these results indicate that HPC and HSC show different mobilization kinetics in response to FL, resulting in preferential mobilization of HPC at day 5, followed by HSC mobilization at day 10, allowing for the use of FL as a selective mobilizing agent.

## AUTHORSHIP AND DISCLOSURES

EK and MP: conception and design of the study, collection, analysis and interpretation of the data and writing of the paper; HH: collection, analysis and interpretation of the data; GV: conception and design of the study and collection of the data; WF: conception and design of the study and critical revision of the paper. All authors approved the final version of the manuscript. The authors reported no potential conflicts of interest.

## ACKNOWLEDGEMENTS

The authors would like to thank J.M.E. van den Oever for technical assistance.

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# CHAPTER 3

## REPEATED HEMATOPOIETIC STEM AND PROGENITOR CELL MOBILIZATION WITHOUT DEPLETION OF THE BONE MARROW STEM AND PROGENITOR CELL POOL IN MICE FOLLOWING REPEATED ADMINISTRATION OF RECOMBINANT MURINE G-CSF

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Published in: Human Immunology. 2007; 68: 368-74

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# ABSTRACT

Administration of recombinant-human G-CSF (rhG-CSF) is highly efficient in mobilizing hematopoietic stem and progenitor cells (HSC/HPC) from the bone marrow (BM) towards the peripheral blood. This study was designed to investigate whether repeated G-CSF-induced HSC/HPC mobilization in mice could lead to a depletion of the bone marrow HSC/HPC pool with subsequent loss of mobilizing capacity. To test this hypothesis Balb/c mice were treated with a maximum of 12 repeated 5-day cycles of either 10 µg rhG-CSF/day or 0,25 µg rmG-CSF/day. Repeated administration of rhG-CSF lead to strong inhibition of HSC/HPC mobilization towards the peripheral blood and spleen after > 4 cycles due to the induction of anti-rhG-CSF-antibodies. In contrast, following repeated administration of rmG-CSF, HSC/HPC mobilizing capacity remained intact for up to 12 cycles. The number of CFU-GM per femur did not significantly change for up to 12 cycles. We conclude that repeated administration of G-CSF does not lead to depletion of the bone marrow HSC/HPC pool.

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# INTRODUCTION

Mobilization of hematopoietic stem and progenitor cells (HSC/HPC) to facilitate bone marrow reconstitution following myeloablative therapy can be achieved by administration of cytokines, chemokines, chemotherapy, or a combination of these agents.<sup>1-3</sup> Combining chemotherapy and hematopoietic growth factors can enhance mobilizing capacity very effectively and has become a routine procedure in the mobilization of peripheral blood HSC/HPC for transplantation. Nowadays, administration of a single growth factor has become the standard regimen in peripheral blood HSC/HPC mobilization in healthy allogeneic stem cell donors. The most commonly used growth factor is recombinant-human-G-CSF (rhG-CSF), which is capable of increasing the number of circulating HSC/HPC in peripheral blood approximately 10- to 100-fold, following administration for 5 subsequent days.<sup>4</sup> Although widely used in clinical practice, the events that lead to HSC/HPC mobilization after G-CSF administration are incompletely understood. Under physiological conditions HSC/HPC are retained in the bone marrow by adhesive and chemotactic interactions, such as the interactions between VLA-4/VCAM-1 and between CXCL12/CXCR4.<sup>5</sup> Upon administration of G-CSF, proteases like neutrophil elastase (NE) and cathepsin-G (CG) accumulate in the bone marrow and degrade the adhesive interactions between HSC/HPC and their micro-

environment.<sup>5-9</sup> In addition, studies in rhesus monkeys indicated a critical role for the matrix metalloproteinase-9 (MMP-9; gelatinase B) that is stored in the azurophilic granules of neutrophils.<sup>10</sup> Recently, it was shown that administration of G-CSF leads to a decrease in *serpina1* ( $\alpha_1$ -antitrypsin) and *serpina3* ( $\alpha_1$ -chymotrypsin) protein concentrations in the bone marrow, but not in the liver, leading to a shift in the balance between serine proteases and their inhibitors in the bone marrow.<sup>9</sup> This resulted in the accumulation of active NE and CG in the bone marrow and the cleavage and inactivation of the molecules responsible for the retention of HSC/HPC in the bone marrow micro-environment.<sup>9</sup> Recently, we have shown that low-dose total body irradiation in mice induces an increase of *serpina1* in the bone marrow, leading to a complete inhibition of IL-8 and G-CSF-induced HSC/HPC mobilization.<sup>11</sup>

The kinetics of G-CSF induced HSC/HPC mobilization differs from mobilization induced by other cytokines, e.g. IL-1 and IL-8, which are faster acting and show optimal mobilization after several hours and 20-30 minutes upon administration respectively.<sup>12,13</sup> Following G-CSF administration in mice, the highest numbers of HSC/HPC are reached after 4-5 days.<sup>1</sup> However, the effect of repeated administration of G-CSF on HSC/HPC mobilization has not been studied thus far. The present study was designed to examine whether repeated G-CSF administration could lead to a depletion of the bone marrow HSC/HPC pool with subsequent loss of mobilizing capacity. To test this hypothesis, mice were treated with repeated cycles of either rhG-CSF or recombinant murine G-CSF (rmG-CSF). We report that repeated administration of rhG-CSF leads to strong inhibition of HSC/HPC mobilization due to the induction of anti-rhG-CSF-antibodies. In contrast, following repeated administration of rmG-CSF, HSC/HPC mobilizing capacity remains intact without depletion of the bone marrow HSC/HPC pool.

## MATERIALS AND METHODS

### MICE

Female Balb/c mice with an age ranging between 8-12 weeks were purchased from Charles River Laboratories (Maastricht, The Netherlands). The animals were fed commercial rodent food and acidified water ad libitum. The institutional ethical committee on animal experiments approved all experimental protocols.

### ADMINISTRATION OF RECOMBINANT-HUMAN-G-CSF AND MURINE G-CSF

Mice were injected intraperitoneally (i.p.) with either 10  $\mu$ g recombinant-methionyl-human-G-CSF (rhG-CSF, filgrastim, Amgen, Thousand Oaks, California, USA) in 0.2 ml Phosphate Buffered Saline/0.1% Bovine Serum Albumin (designated as PBS

throughout the rest of the manuscript) or with 0.25 µg recombinant-murine-G-CSF (rmG-CSF, kindly provided by Amgen, Thousand Oaks, California, USA) in 0.2 ml PBS. The doses were chosen to achieve optimal levels of peripheral blood HSC/HPC mobilization based on previous experiments (data not shown). Mice injected with 0.2 ml PBS served as controls. Each mobilization cycle consisted of injections given on 5 consecutive days with a 9-day rest period until the next mobilization cycle. In a separate experiment mice were subsequently treated with 30 µg IL-8 (Novartis, Vienna, Austria) as a single i.p. injection, 24 hours after the final dose of rhG-CSF was administered.

## PREPARATION OF CELL SUSPENSIONS AND BONE MARROW EXTRACELLULAR EXTRACTS

At the end of the mobilization protocol mice were killed by CO<sub>2</sub> asphyxiation. Peripheral blood was obtained by intracardiac puncture and cell counts were performed on a Sysmex<sup>®</sup> F800 counter (TOA Medical Electronics Co. LTD, Kobe, Japan). Peripheral blood-derived mononuclear cell suspensions (MNC) were obtained by Ficoll separation as described previously.<sup>10</sup> Bone marrow cells were harvested by flushing a femur under sterile conditions with RPMI 1640, containing 500 µg/ml penicillin, 250 µg/ml streptomycin, 1% heparin and 2% fetal bovine serum (FBS, Gibco, Grand Island, NY). Spleen cells were obtained by straining the spleen through a 100 µm pore filter (BD Falcon, Franklin Lakes, New Jersey, USA) and washing the cell suspension twice in a RPMI 1640 solution containing penicillin, streptomycin and FBS, as described above. 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.

## PROGENITOR CELL ASSAY

Colony Forming Units - Granulocyte Macrophage (CFU-GM) were cultured as described previously.<sup>10</sup> Briefly, 5 x 10<sup>5</sup> peripheral blood cells, 5 x 10<sup>4</sup> bone marrow cells or 1 x 10<sup>6</sup> spleen cells were resuspended in a 3.5 cm<sup>2</sup> Petri dish containing 1 ml of a 40% methylcellulose, 30% IMDM and 20% FCS solution with the addition of 1.25 ng/ml recombinant murine Granulocyte-Macrophage-Colony-Stimulating-Factor (GM-CSF). After 7 days of incubation at a temperature of 37 °C in a 5% CO<sub>2</sub> environment colonies (i.e. a cluster of 20 or more cells) were scored using an inverse light microscope.

## FACS ANALYSIS OF PERIPHERAL BLOOD AND BONE MARROW SAMPLES

To determine the absolute number of neutrophils in peripheral blood and bone marrow samples, immunophenotyping was performed as follows. First, erythrocytes were lysed by incubation with NH<sub>4</sub>Cl-buffer for 10 minutes at 4

°C. Next, the leukocytes were washed twice with PBS, containing 0.1% GPO (Pasteurized Plasma Protein Solution, Sanquin CLB, Amsterdam, The Netherlands). Subsequently,  $2 \times 10^5$  cells were incubated with Phycoerythrin (PE)-conjugated CD3 (clone 145-2C11), CD45R/B220 (clone RA3-6B2) and FITC-conjugated GR-1 (clone RB6-8C5) (Pharmingen, San Diego, CA, USA) for 30 minutes at 4 °C. After washing, the cells were incubated at 4 °C with Cy-chrome conjugated anti-Ly-5 antibody for 30 minutes. The cells were then washed twice and the relative fluorescence intensity was measured by FACScan analysis (Becton & Dickinson, Mountain View, CA, USA). The number of GR-1<sup>high</sup>-CD3<sup>neg</sup>-B220<sup>neg</sup> cells within the Ly-5<sup>+</sup> population was determined to represent mature neutrophils.

### ANTI-RHG-CSF-ANTIBODY ASSAY

To measure anti-rhG-CSF-antibodies in peripheral blood plasma, a 96-wells MaxiSorb-plate was coated with 5 µg/ml rhG-CSF in PBS by overnight incubation at 4 °C. Next, the plates were washed 5 times with PBS/0.1% Tween. Plasma samples were diluted 500 times in PBS/0.1% Tween/1% BSA and added to the wells. After overnight incubation at 4 °C the plates were washed 5 times, incubated with goat-anti-mouse IgG HRP-conjugate in a 5000 x dilution at room temperature. After 2 hours 100 µl of the substrate solution (10.8 ml H<sub>2</sub>O, 1.2 ml 1.1 M Na-Acetate, 100 µl Tetramethyl Benzidine (6.0 mg/ml in 100% DMSO) and 5 µl H<sub>2</sub>O<sub>2</sub>) were added to each well, after which the assay was blocked by adding 1.0 M H<sub>2</sub>SO<sub>4</sub>. The OD was determined at 450 nm by a spectrophotometer.

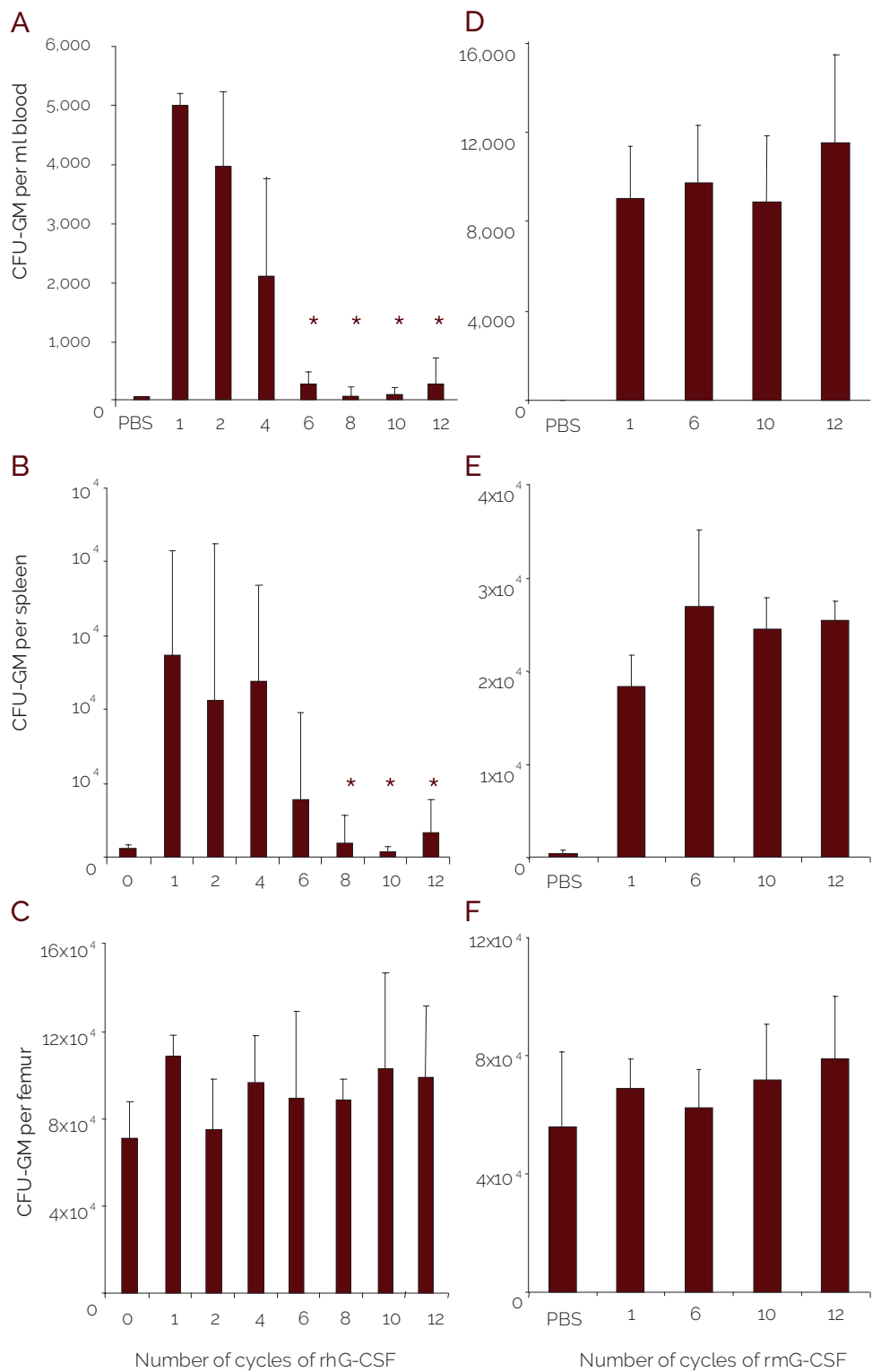
### STATISTICAL ANALYSIS

Differences between the different treatment groups were evaluated using the Student's *t*-test. P-values of <0.05 were considered statistically significant. All data are expressed as mean ± SD.

## RESULTS

### HSC/HPC-MOBILIZATION IS SIGNIFICANTLY REDUCED AFTER REPEATED CYCLES OF RHG-CSF ADMINISTRATION

To assess the effect of repeated rhG-CSF injections on HSC/HPC mobilization, cohorts of mice were given 10 µg rhG-CSF (n=5) or PBS (n=5) i.p., daily for 5 consecutive days, followed by a 9-day rest period. This was repeated 2, 6, 8, 10 and 12 times. A single cycle of 5 days of either rhG-CSF or PBS was administered to age-matched controls. After the final cycle, the mice were sacrificed and the number of CFU-GM was analyzed in peripheral blood, spleen and bone marrow. In mice treated with 2 cycles of rhG-CSF, HSC/HPC mobilization towards the peripheral blood was



not significantly different from the control group that received 1 cycle of rhG-CSF ( $p=0.23$ , **Figure 1A**). In contrast, mice treated with 4 cycles of rhG-CSF showed on average a 58% decrease in peripheral blood HSC/HPC mobilization compared to controls. Furthermore, peripheral blood HSC/HPC mobilization in mice treated with 6, 8, 10 or 12 cycles of rhG-CSF was decreased to levels comparable to control mice treated with PBS alone ( $p<0.001$ ; **Figure 1A**).

HSC/HPC mobilization towards the spleen did not differ significantly between mice receiving 1, 2, 4 or 6 cycles of rhG-CSF ( $p>0.12$  for all groups, **Figure 1B**). However, following 8, 10 or 12 cycles of rhG-CSF, HSC/HPC mobilization towards the spleen was significantly decreased to a level comparable to mice treated with PBS alone ( $p=0.01$  for 12 vs. 1 cycle(s), **Figure 1B**).

Bone marrow CFU-GM were determined to define whether the decrease in HSC/HPC mobilization towards peripheral blood and the spleen following multiple cycles of rhG-CSF was caused by a decrease in the bone marrow HSC/HPC pool. However, no significant difference was found between the numbers of CFU-GM per femur in the experimental groups compared to PBS treated controls ( $p>0.06$  for all groups, **Figure 1C**). Together, these results show that HSC/HPC mobilization is significantly decreased following  $\geq 6$  cycles of rhG-CSF administration, whereas the number of progenitor cells in the bone marrow remained unaffected.

### BONE MARROW NEUTROPHILS ARE NOT AFFECTED BY MULTIPLE CYCLES OF RHG-CSF ADMINISTRATION

Bone marrow neutrophils play a central role in G-CSF-induced HSC/HPC mobilization.<sup>5,6</sup> To investigate whether the absence of HSC/HPC mobilization following multiple cycles of rhG-CSF could be explained by a shift in neutrophil numbers, bone marrow cells obtained from mice that underwent multiple cycles of rhG-CSF-induced mobilization were analyzed by flow cytometry. A decrease was observed in the absolute number of GR-1<sup>high</sup>-CD3<sup>neg</sup>-B220<sup>neg</sup> cells following 6 or more cycles of rhG-CSF, but no statistical significance was reached when

#### < **FIGURE 1** CFU-GM in peripheral blood, spleen and femur after repeated cycles of rhG-CSF and rmG-CSF.

*Mice were given 10  $\mu$ g rhG-CSF or 0.25  $\mu$ g rmG-CSF daily for 5 days, followed by a 9-day rest period (pooled data from 2 identical experiments;  $n = 5$  mice per group). Each cycle was repeated 2, 6, 7, 8, 10 and 12 times for the rhG-CSF group or 1, 6, 10 and 12 times for the rmG-CSF group respectively. A control group was given PBS ( $n = 5$  for each group). On day 5 of the final cycle peripheral blood, spleen and bone marrow were harvested and CFU-GM were determined in peripheral blood, spleen and bone marrow for (A-C) rhG-CSF treated mice and (D-F) rmG-CSF treated mice respectively. (\*  $p < 0.05$  vs. 1 cycle).*

these groups were compared to 1 cycle of rhG-CSF (Figure 2). This shows that the absence of HSC/HPC mobilization could not be explained by a change in the number of neutrophils following multiple cycles of rhG-CSF.

### ABSENCE OF HSC/HPC MOBILIZATION IS NOT DUE TO A DEFECT IN MOBILIZING CAPACITY

To determine whether the decrease in HSC/HPC mobilization was specific for repeated rhG-CSF administration or the consequence of a general inability to mobilize HSC/HPC, mice were treated with a single injection of 30  $\mu$ g IL-8, 24 hours after the final rhG-CSF dose was administered. Following IL-8 treatment of mice that had been treated with 12 cycles of rhG-CSF, a 10-15-fold increase in CFU-GM per ml peripheral blood was observed, comparable to the increase in CFU-GM per ml peripheral blood that was observed in an age-matched control group that had not been treated with rhG-CSF previously (data not shown). Therefore, the inability of HSC/HPC to mobilize in response to rhG-CSF administration was a consequence of repeated rhG-CSF administration and not due to a general defect in HSC/HPC mobilization.

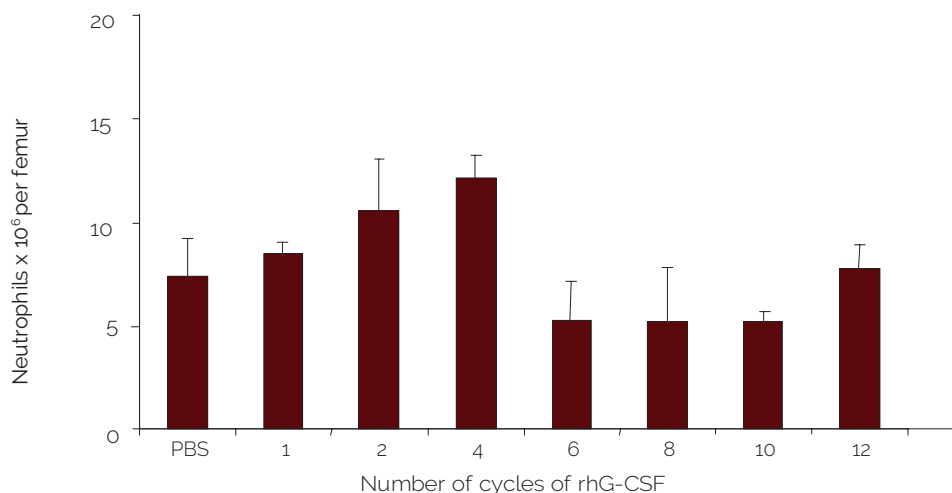


**FIGURE 2** Absolute number of bone marrow neutrophils per femur after repeated cycles of rhG-CSF.

Bone marrow cells were harvested from the femurs of groups of mice ( $n = 5$  mice per group) after 1-12 cycles of rhG-CSF. Age-matched control mice ( $n = 5$ ) receiving only PBS served as controls. The number of neutrophils was determined by immunophenotyping and is expressed as  $GR-1^{high}-CD3^{neg}-B220^{neg}$  cells x 10<sup>6</sup> per femur.

### NEUTRALIZING ANTI-RHG-CSF-ANTIBODIES ARE INDUCED FOLLOWING REPEATED CYCLES OF RHG-CSF

To study whether the absence of HSC/HPC mobilization following 6 or more cycles of rhG-CSF was caused by the formation of neutralizing antibodies specific for rhG-CSF, peripheral blood plasma was analyzed. In mice treated with 6 or more cycles of rhG-CSF, anti-rhG-CSF-antibodies were significantly increased compared to control mice (anti-rhG-CSF antibody level (in arbitrary units)  $3.93 \pm 2.55$ ,  $21.36 \pm 10.51$ ,  $44.78 \pm 6.39$ ,  $38.89 \pm 14.24$  after 1, 2, 6 and 12 cycles of rhG-CSF,  $p=0.005$  for 1 versus 12 cycles, **Figure 3**). As a control, mice were treated with rmG-CSF for up to 12 cycles. In these mice no significant change in the relative amount of plasma antibodies was observed.

### REPEATED ADMINISTRATION OF MURINE G-CSF DOES NOT LEAD TO DEPLETION OF THE BONE MARROW HSC/HPC POOL

Since repeated administration of rhG-CSF lead to the induction of anti-rhG-CSF-antibodies and a subsequent loss of mobilizing capacity, the effect of repeated mobilization on depletion of the bone marrow HSC/HPC pool was investigated by using rmG-CSF as a mobilizing cytokine. Cohorts of mice were given  $0.25 \mu\text{g}$

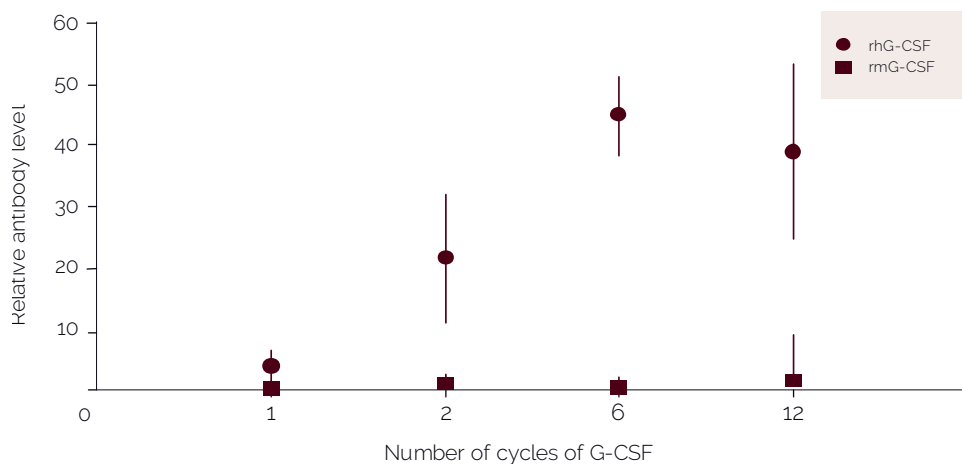


**FIGURE 3** Anti-rhG-CSF-antibodies in plasma samples of mice treated with repeated cycles of rhG-CSF or rmG-CSF.

*In mice treated with repeated cycles of rhG-CSF (●) or rmG-CSF (■) the amount of anti-rhG-CSF-antibodies was determined by means of anti-rhG-CSF-antibody assay. Relative antibody levels are corrected for background antibody detection in PBS treated mice and are expressed as the ratio ( $\pm$  SD) between antibody levels measured in mice treated with > 1 cycle of rhG-CSF or rmG-CSF and antibody levels in a control group treated with 1 cycle of rhG-CSF.*

rmG-CSF (n=5) or PBS (n=3) as a control i.p. for 5 days, followed by a 9-day rest period. This was repeated for 6, 10 and 12 cycles. After the final cycle of rmG-CSF administration, mice were sacrificed and peripheral blood and spleen cells were harvested. Repeated cycles of rmG-CSF did not significantly decrease mobilization of HSC/HPC towards the peripheral blood and spleen compared to mice receiving 1 cycle of rmG-CSF (CFU-GM per ml peripheral blood:  $22.15 \pm 3.04$ ,  $9019 \pm 4746$ ,  $9771 \pm 2557$ ,  $9771 \pm 2557$  and  $8885 \pm 2893$  following PBS and 1, 6, 10 and 12 cycles of rmG-CSF respectively, **Figure 1D**; CFU-GM per spleen:  $348.05 \pm 446.82$ ,  $18.4 \times 10^3 \pm 3.34 \times 10^3$ ,  $26.9 \times 10^3 \pm 8.27 \times 10^3$  and  $25.5 \times 10^3 \pm 2.1 \times 10^3$  following PBS and 1, 6, 10 and 12 cycles of rmG-CSF respectively, **Figure 1E**). These data indicate that repeated rmG-CSF administration does not lead to inhibition of HSC/HPC mobilization. The number of HSC/HPC in the bone marrow was not affected by repeated administration of rmG-CSF: no significant difference was found in CFU-GM per femur in rmG-CSF mobilized versus PBS treated mice (CFU-GM per femur:  $5.57 \times 10^4 \pm 2.53 \times 10^4$ ,  $6.88 \times 10^4 \pm 9.87 \times 10^3$ ,  $6.23 \times 10^4 \pm 1.31 \times 10^4$ ,  $7.16 \times 10^4 \pm 1.86 \times 10^4$  and  $7.88 \times 10^4 \pm 2.14 \times 10^4$  following PBS and 1, 6, 10 and 12 cycles of rmG-CSF respectively, **Figure 1F**).

## DISCUSSION

Cytokine-induced stem cell mobilization has become a routine procedure to facilitate clinical HSC transplantation. Although G-CSF is the most widely used cytokine for these purposes, relatively little is known about the effect of G-CSF on the size of the HSC/HPC pool in stem cell donors. Long-term administration of rhG-CSF to humans does not lead to neutropenia or to a decrease in the biological response towards rhG-CSF, but the effects of administration of G-CSF on the stem cell pool in the bone marrow remain unresolved.<sup>14</sup> Theoretically, repeated HSC/HPC mobilization may lead to a depletion of stem cells from the bone marrow, since after multiple cycles of G-CSF all HSC/HPC may be recruited to the peripheral blood and a proportion may undergo apoptosis.<sup>15</sup> This would ultimately lead to depletion of the bone marrow stem cell pool with subsequent bone marrow hypoplasia and pancytopenia.

In the present study, we have addressed this issue by investigating the effect of administration of recombinant-human-G-CSF and recombinant-murine-G-CSF for repeated intervals in a murine model. Administration of 6 or more cycles of rhG-CSF leads to a loss of mobilizing capacity towards the peripheral blood and spleen caused by the induction of neutralizing anti-rhG-CSF antibodies which have been developed after more than 6 cycles of rhG-CSF. In contrast, in mice treated with

rmG-CSF, no anti-rhG-CSF-antibodies were found, indicating that the antibodies that are induced were specific for rhG-CSF.

Recombinant-human-G-CSF differs from endogenous murine G-CSF with respect to the amino-acid sequence of the molecule, as there is only a 72,8% homology between the two cytokines.<sup>16</sup> This difference may be responsible for the induction of anti-rhG-CSF-antibodies following repeated rhG-CSF administration in mice. Adverse effects due to the administration of heterologous hematopoietic growth factors were first reported by Garcia et al. when immunological neutralization of various erythropoietins lead to profound anemia in a rabbit model.<sup>17</sup> It was hypothesized that high titers of antibodies directed against human erythropoietin were responsible for this phenomenon. Furthermore, administration of murine G-CSF, chemically conjugated to either keyhole limpet hemocyanin or ovalbumin, resulted in the endogenous production of murine G-CSF-specific auto-IgG responses in mice that lasted for more than 9 months.<sup>18</sup> In dogs, the spontaneous induction of canine G-CSF cross-neutralizing IgG responses after the administration of rhG-CSF showed that prolonged neutropenia could occur after administration of a xenogeneic hematopoietic growth factor.<sup>19</sup> However, in the study described above no results were shown on the mobilizing capacity in these animals. In humans, prolonged continuous treatment with G-CSF in patients with severe congenital neutropenia (Kostmann syndrome) or acquired neutropenia has not resulted in a decreased biological response, showing that rhG-CSF is not immunogenic in humans.<sup>14</sup> However, when used repeatedly in a murine model, a decreased biological response is observed due to cross-species reactivity. In future mouse models of repeated HSC/HPC mobilization, the use of a species-specific cytokine like rmG-CSF is preferable.

Recently, the role of neutrophils in rhG-CSF-induced HSC/HPC-mobilization has been emphasized by the finding that bone marrow neutrophils are the source of proteases that disrupt HSC/HPC-stromal cell interactions during mobilization.<sup>5,11-13</sup> In addition it has been shown previously that HSC/HPC mobilization was not dependent on G-CSF-receptor expression on HPC, but rather on the number of functional neutrophils.<sup>20-22</sup> In the current study, no significant effect of repeated rhG-CSF administration was found on the amount of neutrophils in the bone marrow (expressed as the number of GR-1<sup>high</sup>-CD3<sup>neg</sup>-B220<sup>neg</sup> cells), suggesting that the amount of bone marrow neutrophils did not play an important role in the decrease of mobilization after repeated rhG-CSF administration.

Since HSC/HPC mobilization was absent in mice treated with rhG-CSF, the effect of repeated HSC/HPC mobilization could not be studied in this model. Therefore, in an identical set of experiments, mice were treated with repeated cycles of rmG-CSF, which is non-immunogenic in mice. Repeated administration of up to 12 cycles of rmG-CSF did not result in a loss of mobilizing capacity of HSC/HPC towards the

peripheral blood. RmG-CSF appeared to be less effective with regard to HSC/HPC mobilization towards the spleen as compared to rhG-CSF. The underlying mechanism for this phenomenon is unclear, but it is hypothesized that the xenogeneic nature of rhG-CSF, when administered in mice, plays a role. However, the amount of CFU-GM per spleen remained unchanged over time as opposed to a gradual decrease after > 4 cycles of rhG-CSF. Furthermore, repeated administration of rmG-CSF did not result in a reduction of the absolute number of CFU-GM per femur. Since it was shown in previous studies that in peripheral blood the number of CFU-GM adequately reflects the number of stem cells, we used the number of CFU-GM per femur as a marker for the number of stem cells in the bone marrow.<sup>12,13</sup> We therefore conclude from these experiments that repeated administration of rmG-CSF does not lead to depletion of the bone marrow HSC/HPC pool. However, the results from our study do not rule out other short- or long-term effects of G-CSF administration, e.g. due to functional impairment of immune regulation.<sup>23</sup> In conclusion, we have shown that repeated administration of rhG-CSF leads to a loss of mobilizing capacity towards the peripheral blood and spleen in mice, due to the induction of anti-rhG-CSF-antibodies in the plasma of these mice. Repeated HSC/HPC mobilization through the administration of rmG-CSF does not lead to depletion of the bone marrow HSC/HPC pool.

## ACKNOWLEDGEMENTS

This study is financially supported by a grant from the Dutch Cancer Society (NKB; grant no. RUL2002-2720).

Dr Molineux is employed by Amgen whose products were used in the present study.

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# CHAPTER 4

## PERIPHERAL BLOOD HEMATOPOIETIC STEM AND PROGENITOR CELL FREQUENCY IS UNCHANGED IN PATIENTS WITH ALPHA-1-ANTITRYPSIN DEFICIENCY

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Published in: *Int. J. Hematology*. 2014; 99: 714-20

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# ABSTRACT

Granulocyte-colony-stimulating factor (G-CSF)-induced hematopoietic stem and progenitor cell (HSPC) mobilization is associated with the release of neutrophil-derived proteases. Previously, we have shown in mice that alpha-1-antitrypsin (AAT) inhibits these proteases, resulting in inhibition of HSPC mobilization. Here we studied the relationship between AAT and HSPC in steady state and cytokine-induced mobilization in humans. Patients with alpha-1-antitrypsin deficiency (AATD) have an 85-90% decrease of AAT in the peripheral blood (PB). We hypothesized this leads to increased proteolytic activity in the bone marrow and increased steady state PB HSPC numbers. Using flow cytometry and semi-solid cell culture, we found no significant difference in PB HSPC in AATD patients (n=18) as compared to controls (n=22). Healthy stem cell donors (n=43) were mobilized with G-CSF for 5 days and the number of CD45<sup>+</sup>/CD34<sup>+</sup> HSPC were determined in PB. We found that, during mobilization, PB AAT levels increased significantly, positively correlating with PB CD45<sup>+</sup>/CD34<sup>+</sup> cells ( $r=0.31$ ,  $p=0.005$ ).

In conclusion, although serum AAT levels and HSPC mobilization in healthy stem cell donors are positively correlated, AAT is not an indispensable protease-inhibitor in the constitutive circulation of HSPC. These findings suggest a model in which both protease-dependent and -independent pathways contribute to HSPC mobilization.

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# INTRODUCTION

Granulocyte-colony-stimulating factor (G-CSF) mobilized stem and progenitor cells (HSPC) are routinely used as a source for clinical stem cell transplantation. In recent years, research has started to unravel the mechanisms underlying cytokine-induced HSPC mobilization. G-CSF administration induces expansion and activation of hematopoietic progenitor cells in the bone marrow, which leads to increased numbers of neutrophils.<sup>1,2</sup> Upon activation, neutrophils release proteases, including neutrophil elastase (NE), cathepsin G (CG) and matrix metalloproteinase-9 (MMP-9).<sup>3-6</sup> These proteases degrade adhesive interactions between HSPC and their microenvironment, including CXCR4/CXCL12, VLA-4/VCAM-1 and c-KIT/SCF.<sup>5,7,8</sup> Levels of CXCL12, a chemokine that attracts HSPC, are down regulated in the bone marrow, which induces a gradient, allowing HSPC to mobilize towards the peripheral blood.<sup>8</sup> Furthermore, administration of G-CSF induces apoptosis of trophic endosteal macrophages (osteomacs) that support osteoblast function.<sup>9</sup> These osteoblasts are an important constituent of the stem cell niche and play a

critical role in HSPC maintenance, via soluble and membrane bound factors such as CXCL12 and SCF.<sup>10</sup> During G-CSF-induced HSPC mobilization, expression of alpha-1-antitrypsin (AAT) is strongly reduced in osteoblasts, both at the protein and mRNA levels.<sup>11,12</sup> Alpha-1-antitrypsin is a potent inhibitor of serine proteases, including NE and CG. Decreased levels of AAT are associated with an accumulation of active proteases in the bone marrow compartment, resulting in HSPC mobilization from the bone marrow towards the peripheral blood.<sup>12</sup> Increased levels of AAT, either by induction via low dose irradiation or via intraperitoneal administration of exogenous AAT, result in a near complete inhibition of HSPC mobilization in mice.<sup>13</sup> In the peripheral blood of G-CSF-mobilized healthy individuals, proteases such as MMP-9 and NE, are significantly increased as compared to non-mobilized donors.<sup>14-16</sup> Thus far, peripheral blood levels of AAT during G-CSF-induced HSPC mobilization in humans have not been studied.

The importance of the balance between proteases and protease-inhibitors in retention and mobilization of HSPC suggests that modulation of AAT levels could be a novel strategy to induce HSPC mobilization. To test this hypothesis, we studied the presence of HSPC in the peripheral blood of patients with alpha-1-antitrypsin deficiency (AATD). AATD patients have a mutation in the gene responsible for the expression of AAT, SERPINA1, resulting in severely decreased serum levels of AAT as only 10–15% of AAT is released into the circulation. As a result, the lungs of these patients are exposed to enzymatic damage by proteases such as NE, leading to early onset emphysema and bronchiectasis.<sup>17-19</sup> Such a constitutively decreased AAT level, as observed in AATD patients, could lead to a protease/protease-inhibitor imbalance with elevated protease activity in the bone marrow. Increased proteolytic activity may degrade adhesive interactions between HSPC and their environment and as a result steady state peripheral blood HSPC frequencies could be affected in these patients. Therefore, we determined the number of CD45<sup>+</sup>/CD34<sup>+</sup> cells, as well as the frequency of colony-forming cells in the peripheral blood in a cohort of AATD patients.

In addition, to determine the relationship between serum AAT levels and mobilizing capacity, we investigated HSPC frequencies in the peripheral blood of G-CSF-mobilized healthy donors and correlated this with serum AAT levels.

## MATERIALS AND METHODS

### AATD PATIENTS

During routine follow-up visits to the pulmonology out-patient clinic, blood samples were taken from patients with AATD. All samples were taken between 8 a.m. and 10 a.m. to minimize the possible effect of the circadian rhythm on HSPC mobilization.<sup>20</sup> All patients had a ZZ phenotype (PI\*ZZ) as confirmed by isoelectric focusing. Healthy

volunteers and patients with chronic obstructive pulmonary disease (COPD) levels were used as controls. Individuals with a C-reactive protein (CRP) level  $\geq 5$   $\mu\text{g/ml}$  or non-MM phenotype were excluded from the analysis. Blood samples were analyzed for AAT level, AAT phenotype and HSPC frequency. The levels of two other physiologically important protease-inhibitors, alpha-2-macroglobulin (A2M) and secretory leukocyte proteinase inhibitor (SLPI), were also determined.<sup>21</sup>

### **HUMAN DONORS UNDERGOING G-CSF-INDUCED STEM CELL MOBILIZATION**

Healthy allogeneic peripheral blood stem cell donors were mobilized by injection of recombinant-human-G-CSF (Filgrastim; Amgen, Thousand Oaks, CA), which was given subcutaneously at a dose of 10  $\mu\text{g/kg}$  on days 1–3. On day 4, two doses of 10  $\mu\text{g/kg}$  were given, one in the morning and one in the evening approximately 12 hours before the onset of apheresis on day 5. On days 3, 4 and 5, blood samples were taken prior to G-CSF administration to determine white blood cell counts, neutrophil counts and the frequency of CD34<sup>+</sup> cells, as described previously.<sup>16</sup> Plasma samples were collected to determine AAT levels. All donors gave informed consent and the local ethical committee approved this protocol.

### **DETERMINATION OF PERIPHERAL BLOOD HSPC**

Peripheral blood HSPC were determined by flow cytometry and by HSPC culture. For flow cytometric analysis, peripheral blood mononuclear cells (PBMC) were co-stained with conjugated monoclonal antibodies against CD45 (PE, clone HI30) and CD34 (FITC, clone 8G12) after red blood cell lysis with  $\text{NH}_4\text{Cl}$  (LUMC Pharmacy, Leiden). Antibodies were obtained from BD PharMingen, San Diego, CA. HSPC were defined as the  $\text{FSC}^{\text{lo}}/\text{SSC}^{\text{lo}}/\text{CD45}^+/\text{CD34}^+$  fraction.

To determine the frequency of HSPC by semi-solid cell culture,  $2.0 \times 10^5$  PBMC, obtained after Ficoll separation of heparinized whole blood, were plated in 3.5-cm dishes according to the manufacturer's recommendations (Methocult® H4434 Classic, Stemcell Technologies, Grenoble, France). After 12–14 days of culture in a fully humidified atmosphere of 37°C containing 5%  $\text{CO}_2$ , the number of colonies was scored using an inverted light microscope. Colonies were scored according to the type of progenitors they contained: granulocytes (CFU-G), monocytes (CFU-M), granulocytes and monocytes (CFU-GM), granulocytes, monocytes and erythroid progenitors (CFU-GEMM) or erythroid progenitors only (CFU-E and burst-forming colony (BFU)-E).

### **DETERMINATION OF PLASMA LEVELS OF PROTEASE-INHIBITORS AND AAT PHENOTYPE**

Serum levels of AAT were measured quantitatively by means of radial immunodiffusion using commercially available plates (NOR Partigen, Siemens, Germany).

A2M and SLPI levels were measured using an enzyme-linked immunosorbent assay using a polyclonal rabbit anti-human A2M antibody (DAKO, Glostrup, Denmark) and human anti-SLPI antibody (clone 31, Hycult Biotech, Uden, The Netherlands) respectively.

AAT phenotype was determined by isoelectric focusing; subjects were divided in having a MM-phenotype (normal), ZZ-phenotype (AATD patients) or other phenotype (e.g. MZ or SS).

## STATISTICAL ANALYSIS

Statistical differences were determined using GraphPadPRISM (GraphPad Software, San Diego, CA). Data are presented as mean values  $\pm$  standard error of the mean (SEM). *P* values of  $<0.05$  were considered statistically significant. Spearman's correlation coefficient (*r*) was used to describe the strength of association between two ranked variables.

## RESULTS

### HEMATOPOIETIC STEM CELL FREQUENCIES IN THE PERIPHERAL BLOOD OF AATD PATIENTS AND HEALTHY CONTROLS ARE SIMILAR

To investigate the relationship between serum levels of peripheral blood AAT and steady state peripheral blood HSPC frequencies, patients with AATD were compared with healthy volunteers. To exclude the possibility that the results would be biased by the presence of chronic lung disease alone, a second control group consisting of patients with COPD was included. In total, 20 healthy controls, 21 patients with AATD and 12 patients with COPD were studied. In the healthy control group, 6 subjects were excluded from the analysis ( $n=3$  due to a non-MM phenotype;  $n=3$  due to a CRP level  $\geq 5 \mu\text{g/ml}$ ). In the AATD patient group and in the COPD patient group, 3 and 4 subjects respectively were excluded because of a CRP level  $\geq 5 \mu\text{g per ml}$ . Therefore, 18 patients in the AATD group, 14 healthy controls and 8 COPD patients were available for further analysis. Patient characteristics were comparable, with the exception of age, which was higher in the COPD patient group (Table 1).

As expected, serum AAT levels in AATD patients were significantly decreased compared to both control groups ( $0.21 \pm 0.04 \text{ mg/ml}$  vs  $1.80 \pm 0.32 \text{ mg/ml}$  respectively,  $p<0.0001$ ). No significant differences were observed in A2M or SLPI levels between healthy controls and AATD patients (Figure 1A and 1B).

Subsequently, the absolute number of  $\text{CD45}^+/\text{CD34}^+$  cells was determined by flow cytometry. No significant difference was observed for the AATD patients as compared to both control groups (AATD patients  $3.2 \times 10^{-3} \pm 2.0 \times 10^{-3}/\text{ml}$ , healthy controls  $2.2 \times 10^{-3} \pm 0.9 \times 10^{-3}/\text{ml}$ , COPD patients  $2.2 \times 10^{-3} \pm 2.7 \times 10^{-3}/\text{ml}$ ,  $p=0.12$  for

**TABLE 1** Patient characteristics of AATD patients and control groups.

|                  | n  | Age<br>(years, mean $\pm$ SD) | Age<br>(years, range) | PI phenotype |
|------------------|----|-------------------------------|-----------------------|--------------|
| AATD patients    | 18 | 52.3 $\pm$ 12.2               | 22 - 69               | ZZ           |
| Healthy controls | 14 | 39.3 $\pm$ 11.6               | 22 - 61               | MM           |
| COPD patients    | 8  | 63.9 $\pm$ 6.0                | 55 - 71               | MM           |

comparison between AATD patients and healthy controls,  $p=0.35$  for comparison between AATD patients and COPD patients; **Figure 1C**).

Next, CFU-C frequencies were determined by colony assays in semi-solid cultures. No significant difference was observed between the three groups with respect to the peripheral blood frequency of CFU-G/M/GM/GEMM (AATD patients  $281.0 \pm 178.0$  colonies per  $10^6$  PBMC, healthy controls  $259.5 \pm 75.8$  colonies per  $10^6$  PBMC and COPD patients  $223.4 \pm 96.5$  colonies per  $10^6$  PBMC;  $p=0.67$  for comparison between AATD patients and healthy controls,  $p=0.40$  for comparison between AATD patients and COPD patients; **Figure 1D**). However, the peripheral blood frequency of BFU-E was significantly increased in the AATD patients group as compared to healthy controls (AATD patients  $357.2 \pm 167.8$  colonies per  $10^6$  PBMC, healthy controls  $159.3 \pm 67.1$  colonies per  $10^6$  PBMC), but was lower than BFU-E frequencies in COPD patients ( $612.5 \pm 464.0$  colonies per  $10^6$  PBMC,  $p<0.05$  for comparison between all groups; **Figure 1E**). No significant correlation was found between the serum AAT level, the absolute number of CD45<sup>+</sup>/CD34<sup>+</sup> cells ( $r=-0.18$ ,  $p=0.53$ ) and the total number of CFU-G/M/GM/GEMM ( $r=-0.19$ ,  $p=0.50$ ; data not shown).

In conclusion, very low serum levels of AAT are not associated with changes in steady-state HSPC frequencies in the peripheral blood of patients with AATD.

### AAT SERUM LEVELS ARE INCREASED DURING G-CSF-INDUCED HSPC MOBILIZATION IN HEALTHY HUMAN DONORS

To investigate the effect of G-CSF administration on AAT levels in peripheral blood, 43 healthy human HSPC donors (mean age 40.9 years, range 16-65 years; 26 males, 17 females) were treated with G-CSF for 4 consecutive days. On day 3, 4 and 5, serum AAT levels and CD45<sup>+</sup>/CD34<sup>+</sup> numbers in peripheral blood were determined. In peripheral blood, CD45<sup>+</sup>/CD34<sup>+</sup> numbers increased significantly from  $14.2 \times 10^3$  per ml  $\pm 10.5 \times 10^3$  per ml on day 3 to  $99.9 \times 10^3$  per ml  $\pm 56.0 \times 10^3$  per ml on day 5 ( $p<0.0001$  for day 3 vs day 5; **Figure 2A**). Similarly, AAT levels increased significantly from day 3 to day 5 of G-CSF administration (AAT 140 mg/ml  $\pm 0.20$  mg/ml,

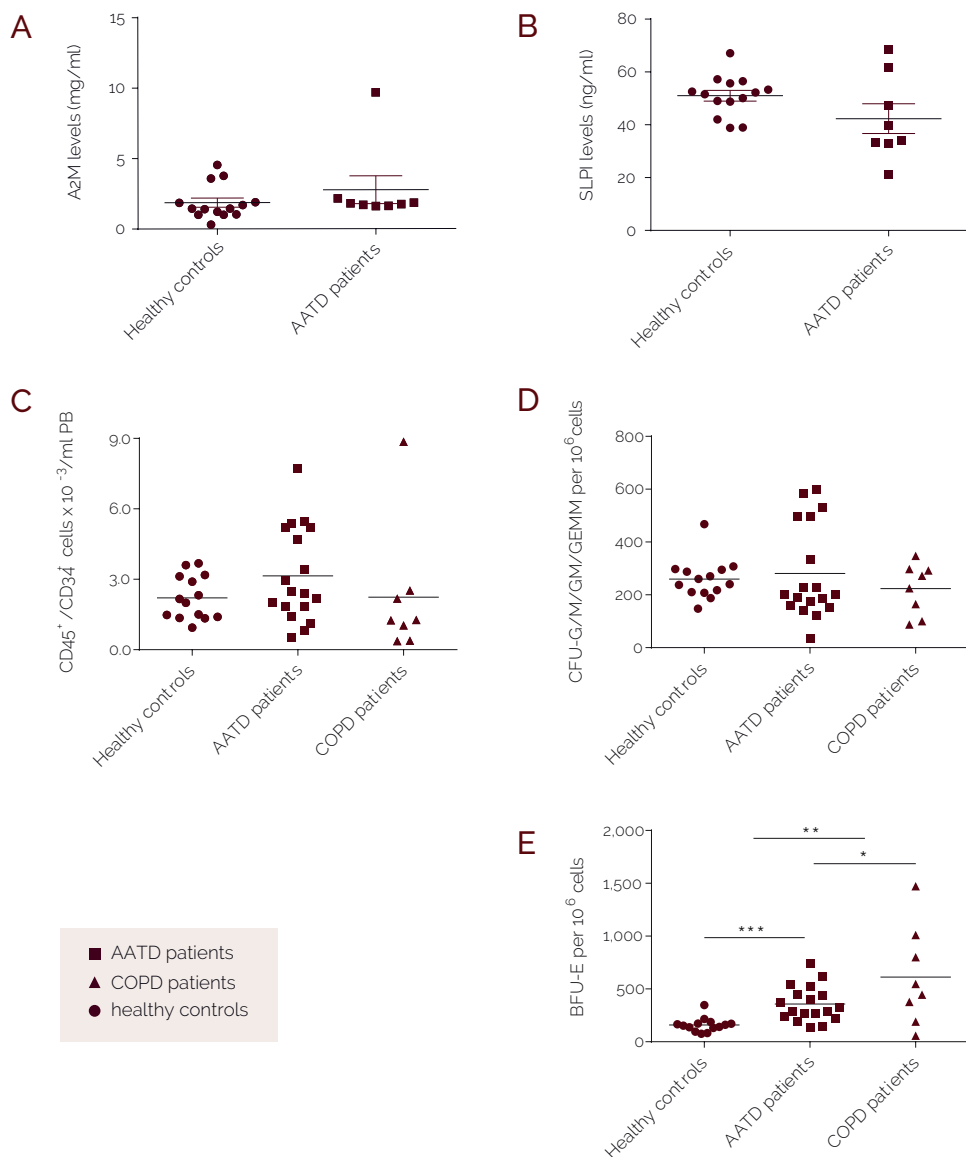


**FIGURE 1** Hematopoietic stem cell frequencies in the peripheral blood of AATD patients are not significantly different as compared to healthy volunteers and patients with COPD.

No significant differences were observed in (A) A2M or (B) SLPI levels in peripheral blood serum obtained from healthy controls and AATD patients. (C) The absolute number of CD45<sup>+</sup>/CD34<sup>+</sup> cells per ml peripheral blood was determined by FACS analysis. AATD patients are compared with healthy controls and COPD patients ( $p \geq 0.10$ ). (D-E) The frequency of HSPC in the peripheral blood was obtained by semi-solid cell culture of  $2.0 \times 10^5$  PBMC. Colonies were manually scored according to the type of progenitors they contained: (D) myeloid (CFU-G/M/GM/GEMM) or (E) erythroid progenitors (BFU-E) (\* $p=0.048$ , \*\* $p=0.016$  and \*\*\* $p=0.0003$ ).

1.47 mg/ml  $\pm$  0.22 mg/ml and 1.54 mg/ml  $\pm$  0.25 mg/ml on day 3, 4 and 5 respectively;  $p < 0.0001$  for day 3 vs day 5; **Figure 2B**). To examine whether CD45<sup>+</sup>/CD34<sup>+</sup> numbers and AAT levels were correlated, a Spearman correlation test was applied. A higher number of peripheral blood CD45<sup>+</sup>/CD34<sup>+</sup> cells was associated with a higher level of serum AAT ( $r = 0.31$ ,  $p = 0.005$ ; **Figure 2C**).

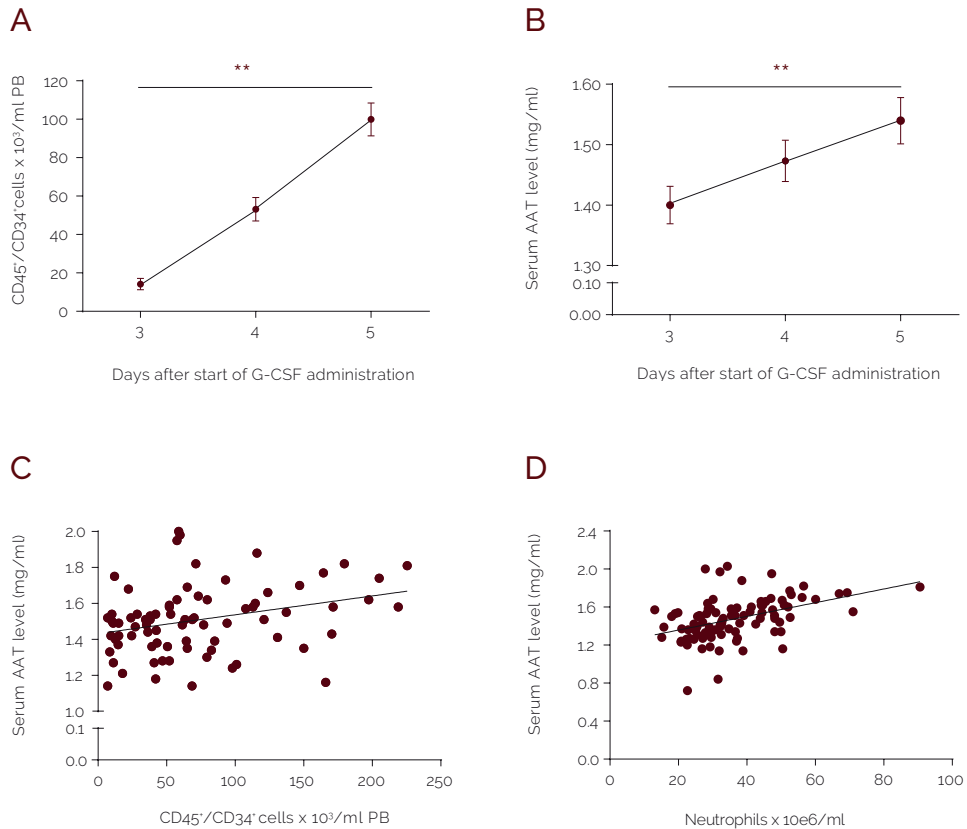


**FIGURE 2** G-CSF-induced peripheral blood HSPC mobilization in human donors in relation to serum alpha-1-antitrypsin levels.

**(A)** Peripheral blood CD45<sup>+</sup>/CD34<sup>+</sup> numbers were determined by flow cytometry in the peripheral blood of human peripheral stem cell donors on day 3, 4 and 5 of G-CSF administration. **(B)** Levels of AAT are measured quantitatively through radial immunodiffusion in the serum of human peripheral stem cell donors on day 3, 4 and 5 of G-CSF administration (\*\* $p < 0.0001$ ). **(C)** CD45<sup>+</sup>/CD34<sup>+</sup> numbers are shown in correlation with serum AAT levels in G-CSF mobilized peripheral stem cell donors ( $r = 0.31$ ,  $p = 0.005$ ). **(D)** Serum AAT levels and neutrophil numbers per ml of peripheral blood are positively correlated at day 5 of G-CSF-induced HSPC mobilization ( $r = 0.49$ ,  $p < 0.0001$ ).

Neutrophil numbers and neutrophil elastase levels in the peripheral blood are positively correlated with the extent of HSPC mobilization.<sup>16</sup> Therefore, we investigated the relationship between neutrophil numbers, neutrophil elastase and AAT levels. An increase in neutrophil numbers is positively associated with a concurrent increase in AAT levels ( $r=0.49$ ,  $p<0.0001$ ; **Figure 2D**). Furthermore, a concomitant increase was observed for white blood cell counts and AAT. No correlation was found for MMP-9 levels and AAT.

In conclusion, G-CSF induced HSPC mobilization in human donors is associated with a concomitant increase in peripheral blood AAT levels between days 3 and 5 of mobilization.

## DISCUSSION

Steady state HSPC content of peripheral blood in healthy persons is relatively low compared to the HSPC content of bone marrow.<sup>22</sup> Peripheral blood HSPC numbers may increase upon internal signals (inflammation, tissue injury) or external signals (cytokines, drugs). We have previously found that HSPC frequencies in the peripheral blood are significantly reduced in germ-free mice and that these numbers increase upon addition of endotoxin to the drinking water.<sup>23</sup> These results indicate that the "constitutive" presence of HSPC in the peripheral blood is actually, at least in part, an induced event, most likely in response to bacterial products. In addition, the level of mobilization is determined by a balance between proteases and their inhibitors, such as AAT.<sup>13</sup> We therefore hypothesized that HSPC frequencies in patients with a deficiency of AAT are increased. However, our study shows that in these patients the frequency of peripheral blood steady state HSPC is not significantly different from controls. The higher number of BFU-E in patients with AATD or COPD most likely reflects a physiological response to a chronic hypoxic condition. The lack of differences in peripheral blood steady state numbers of HSPC could not be explained by a compensatory increase in other protease inhibitors, as A2M or SLPI levels did not significantly differ between healthy controls and AATD patients.

During G-CSF-induced HSPC mobilization in humans, increased levels of proteases are present in the peripheral blood.<sup>16</sup> In the current report, we show that serum AAT levels increase on day 3 to 5 of G-CSF-induced HSPC mobilization in healthy donors. Furthermore, serum AAT levels are positively correlated with HSPC mobilization. The increase in serum AAT levels most likely is a response to the high serum levels of proteases that is observed during G-CSF-induced mobilization in healthy human donors.<sup>16</sup> In this previous study, NE levels increased about 40-fold on day 3 of G-CSF-induced HSPC mobilization as compared to levels at the onset of G-CSF administration. Since AAT binds irreversibly to NE as soon as it is released

into the peripheral circulation, an increase in serum NE during HSPC mobilization will likely induce a concomitant increase in serum AAT. In mice, we have observed a similar phenomenon, as serum levels of AAT increase during G-CSF-induced HSPC mobilization (M.P., unpublished data). The increase of AAT levels in the peripheral blood is in contrast with the decrease of AAT levels that is shown in murine bone marrow during G-CSF induced HSPC mobilization.<sup>12</sup> Similar to what is observed in mice, it can be postulated that in humans AAT levels in the bone marrow are decreased. However, since bone marrow AAT levels were not measured in our study, we were not able to confirm this hypothesis.

During recent years, it became clear that besides the secretion of proteases, other mechanisms also play a role in HSPC mobilization. Mice that are conditionally deficient for one or more proteases including MMP-9, NE and CG mobilize in response to G-CSF.<sup>24</sup> Furthermore, a role has been observed for osteal macrophages and osteoblasts.<sup>9,25,26</sup>

In conclusion, we show that AAT is unlikely to be an indispensable protease-inhibitor in the constitutive circulation of HSPC, suggesting a complex model in which both protease-dependent and -independent pathways may contribute to HSPC mobilization.

## ACKNOWLEDGEMENTS

The authors would like to thank Jan Stolk and Joost van den Muijsenberg for their assistance in obtaining patient data and Bram van der Linden for technical assistance.

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# CHAPTER 5

## MESENCHYMAL STROMAL CELLS INDUCE A PERMISSIVE STATE IN THE BONE MARROW THAT ENHANCES G-CSF-INDUCED HEMATOPOIETIC STEM CELL MOBILIZATION IN MICE

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Published in: Exp. Hematol., 2018;64: 59-70

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# 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.

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## 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 <sup>1,2</sup>).

In the BM, the majority of HSC are found in close proximity to MSC surrounding arterioles and sinusoids.<sup>3-6</sup> MSC-derived CXCL12 and Stem Cell Factor (SCF) are indispensable for HSC maintenance as deletion of either CXCL12 or SCF leads to hematopoietic exhaustion.<sup>7-11</sup> HSC are retained in the niche by adhesion molecules,

including  $\beta 1$ -integrins, interacting with extracellular matrix components and with VCAM, expressed on stromal cells.<sup>12</sup>

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.<sup>13</sup> Another BM-resident macrophage population, expressing CD169, supports the retention of HSC by acting on stromal cells in the niche.<sup>14</sup> Depletion of osteomacs or CD169<sup>+</sup> macrophages results in downregulation of *Cxcl12*, *Vcam*, *Ang-1* and *Scf* and results in subsequent hematopoietic stem and progenitor cell (HSPC) mobilization.<sup>13–15</sup>

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).<sup>16,17</sup> 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.<sup>14,15</sup> 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.<sup>2</sup> When co-transplanted with CD34<sup>+</sup> umbilical cord blood-derived HSPC, MSC enhance both HSC engraftment and hematopoietic recovery.<sup>18,19</sup> 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.<sup>19</sup>

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<sup>2</sup> flask in MSC medium, containing  $\alpha$ MEM (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<sub>2</sub>, 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<sup>3</sup> 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- $\gamma$  (20 ng/ml) or recombinant murine TNF- $\alpha$  (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.<sup>20</sup> Images were acquired and analyzed as previously described.<sup>20</sup> 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  $\mu$ l 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  $\mu$ M 2-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, The Netherlands) respectively. In co-culture experiments, 35 x10<sup>3</sup> 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  $\mu$ m 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<sub>2</sub> 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 °C. 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.<sup>21</sup> 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 °C. The supernatant was stored at -20 °C. To enumerate osteoclasts, 1 x10<sup>5</sup> 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, B220, 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<sub>T</sub>) method, with *Hprt*, *Abl* or *Gapdh* as the endogenous reference genes.

**TABLE 1** Overview of the antibodies that are used throughout the study.

| Antibody         | Label             | Clone    | Company       |
|------------------|-------------------|----------|---------------|
| B220             | Fitc, PerCP-Cy5.5 | RA3-6B2  | BD Pharmingen |
| CD3              | Fitc              | 145-2C11 | BD Pharmingen |
| CD3              | eFluor450         | 145-2C11 | eBioscience   |
| CD4              | Fitc              | GK1.5    | BD Pharmingen |
| CD8              | Fitc              | 53-6.7   | BD Pharmingen |
| CD11b            | biotin, Fitc      | M1/70    | BD Pharmingen |
| CD34             | Alexa Fluor 647   | RAM34    | BD Pharmingen |
| CD45.1           | PE, FITC          | A20      | BD Pharmingen |
| CD45.2           | PerCP-Cy5.5, Fitc | 104      | BD Pharmingen |
| CD68             | PerCP-Cy5.5       | FA-11    | Biolegend     |
| CD115            | BV421             | AFS98    | Biolegend     |
| CD117            | APC-eFluor 780    | 2B8      | eBioscience   |
| CD117            | PE                | 2B8      | BD Pharmingen |
| CD135            | PE                | A2F10.1  | BD Pharmingen |
| CD169            | PE                | 3D6.112  | Biolegend     |
| F4/80            | Fitc, BV510       | BM8      | Biolegend     |
| Gr-1             | APC, Fitc         | RB6-8C5  | BD Pharmingen |
| Ly6C             | APC-Cy7           | AL-21    | BD Pharmingen |
| Ly6G             | APC               | 1A8      | BD Pharmingen |
| Sca-1            | PerCP-Cy5.5       | D7       | eBioscience   |
| MERTK            | PE-Cy7            | DS5MMER  | eBioscience   |
| TER119           | Fitc              | TER-119  | BD Pharmingen |
| Ki67             | PE-Cy7            | B56      | BD Pharmingen |
| Isotype for Ki67 | PE-Cy7            | IgG1     | BD Pharmingen |
| Streptavidin     | Pacific Orange    | -        | Invitrogen    |

**TABLE 2** Overview of the primer pairs that are used throughout the study.

| Gene   | Forward (5'-3')                | Reverse (5'-3')          |
|--------|--------------------------------|--------------------------|
| HPRT   | GGAGCGGTAGCACCTCCT             | AACCTGGTTCATCATCGCTAA    |
| GAPDH  | AAGAGGGATGCTGCCCTTA            | TTGTCTACGGGACGAGGAAA     |
| ABL    | TGGAGATAAACTCTAAGCATAACTAAAGGT | GATGTAGTTGCTTGGGACCCA    |
| CXCL12 | CTGTGCCCTTCAGATTGTTG           | CTCTGCGCCCCTTGTTTA       |
| VCAM-1 | TCTTACCTGTGCGCTGTGAC           | ACTGGATCTTCAGGAATGAGT    |
| SCF    | TCAACATTAGGTCCCGAGAAA          | ACTGCTACTGCTGTCATTCTTAAG |
| Angpt1 | GGAAGATGGAAGCCTGGAT            | ACCAGAGGGATTCCCAAAAC     |
| IL-7   | CTGCTGCAGTCCCAGTCAT            | TCAGTGAGGAATTCCAAAGA     |
| CSF3R  | CTCGACCCCATGGATGTT             | GAGAGACTACATCAGGGCCAAT   |

### 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 duplicate 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<sup>3</sup> 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  $133.7 \pm 3.2$  nm. Typically,  $5.3 \times 10^{10} \pm 1.7 \times 10^{10}$  EV were isolated per  $1 \times 10^6$  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

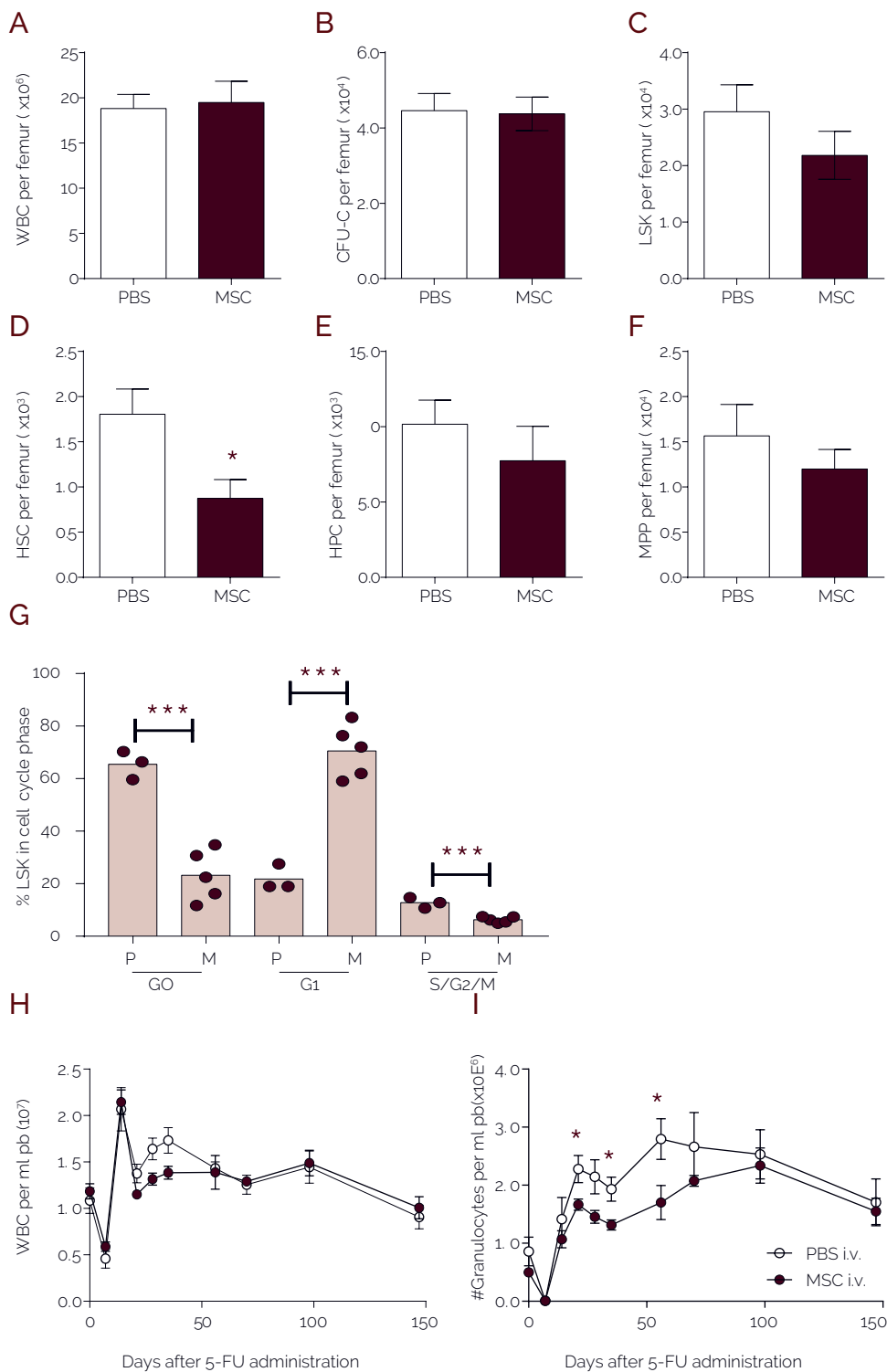
### MSC ADMINISTRATION INCREASES HEMATOPOIETIC STEM – AND PROGENITOR CELL CYCLE ACTIVITY

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<sup>-</sup>Sca-1<sup>c</sup>-Kit<sup>hi</sup>CD34<sup>-</sup>CD135<sup>-</sup>) was significantly decreased (Figure 1D), while the total number of white blood cells (WBC) per femur and the colony-forming capacity of the BM remained comparable to controls (Figure 1A,B). Moreover, there was a trend towards decreased numbers of LSK cells, HPC and MPP per femur (Figure 1C,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<sup>-</sup>Sca-1<sup>c</sup>-Kit<sup>hi</sup> (LSK) cells in G0 and the S/G2/M phase were decreased with 64% and 50.7% compared to PBS controls (Figure 1G). A similar shift in cell cycle activity was observed for HSC and HPC/MPP (supplemental Figure 1A,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 1H,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 1H,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.<sup>14,15</sup> In turn, MSC act on cells of the innate immune system, including macrophages.<sup>22-24</sup> For these reasons, we hypothesized that MSC may alter the HSC niche through macrophages as intermediate cells. Therefore, the presence of osteomacs and CD169<sup>+</sup> macrophages was assessed in BM following MSC administration. A significant decrease in osteomacs and CD169<sup>+</sup> 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**).

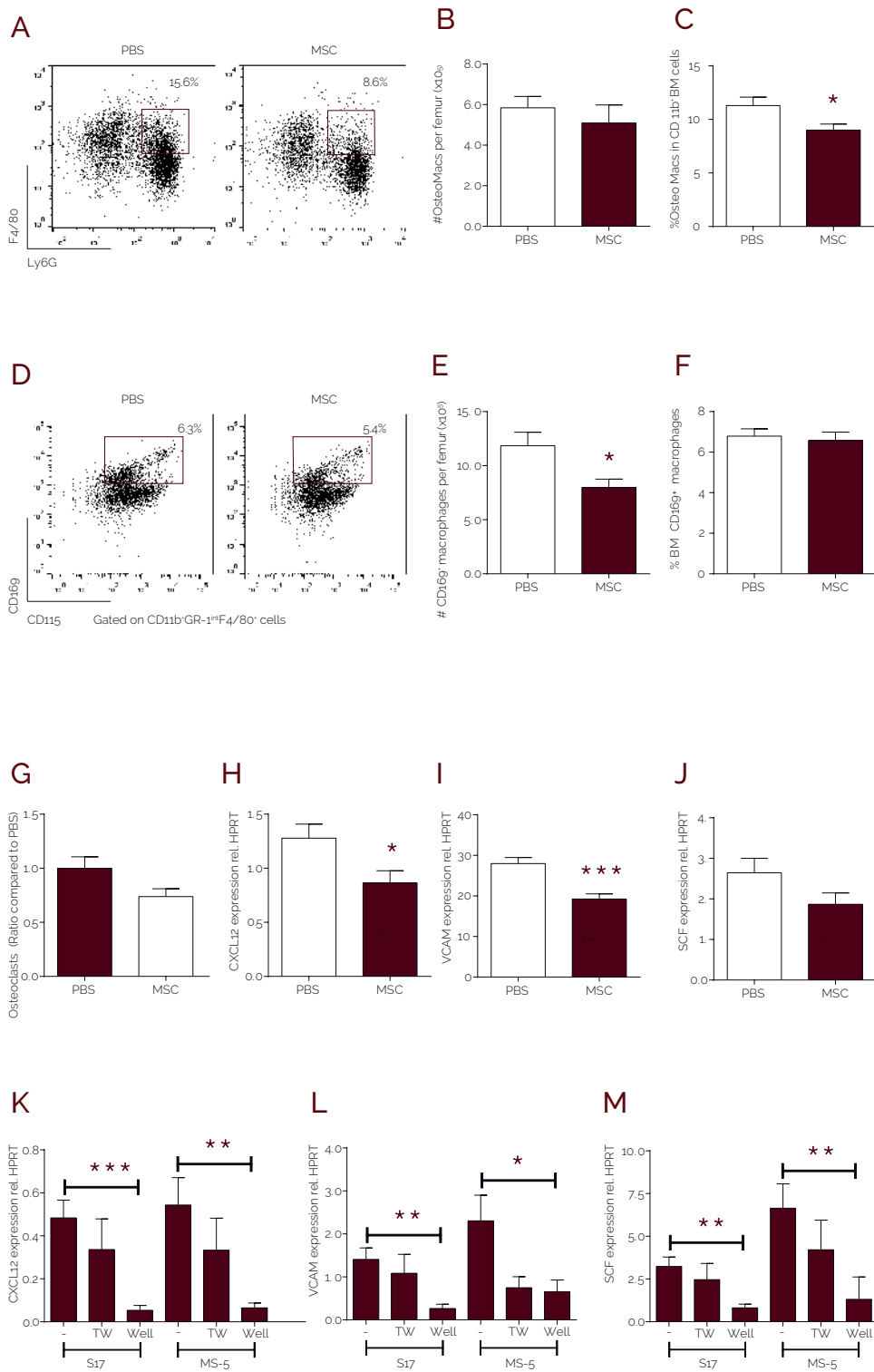
It has been reported that depletion of BM macrophages *in vivo* results in down-regulation of *Cxcl12*, *Vcam*, *Ang-1* and *Scf*.<sup>13-15</sup> Similarly, following MSC administration, the expression of *Cxcl12* and *Vcam* was significantly decreased in endosteal cells, while a modest decrease in *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, these results suggest that MSC administration may impair B lymphopoiesis in the bone marrow.<sup>25</sup>

#### < **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  $\text{Lin}^- \text{Sca-1}^+ \text{c-Kit}^{\text{H}} \text{CD34}^+ \text{CD135}^-$  (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  $\pm$  SEM of 2 separate experiments. \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$  all compared to PBS.



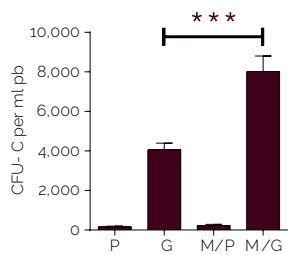
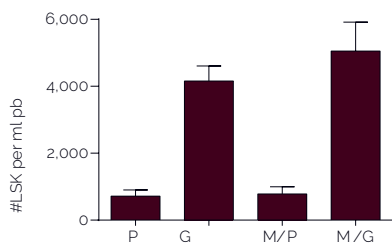
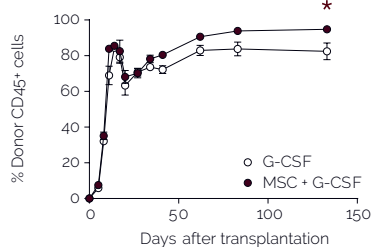
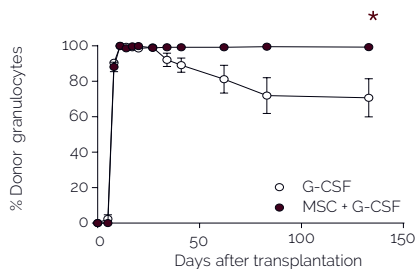
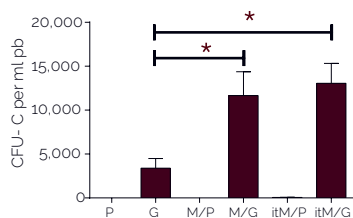
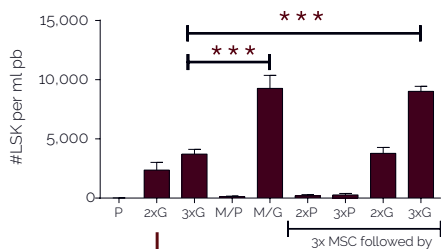
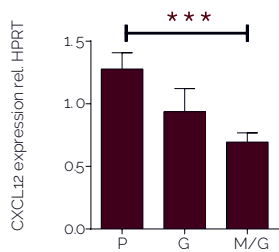
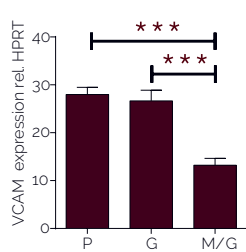
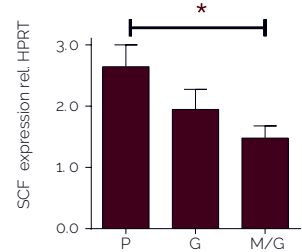
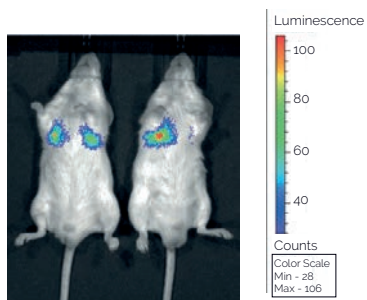
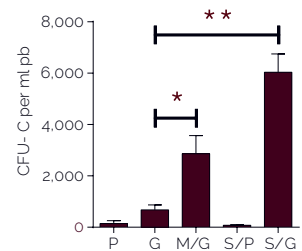
## MSC ENHANCE G-CSF-INDUCED HSPC MOBILIZATION

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.<sup>15,26</sup> 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 S3A). 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.<sup>24</sup> 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- $\gamma$  and TNF- $\alpha$  prior to co-administration with G-CSF. IFN- $\gamma$  and TNF- $\alpha$ -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

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< **FIGURE 2. MSC administration induces downregulation of niche factors in the bone marrow.** (A-C) Osteomacs (n=12), (D-F) CD169<sup>+</sup> 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 separate 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  $\pm$  SEM. \*p<0.05, \*\*p<0.005, \*\*\*p<0.0005 all compared to PBS.

**A****B****C****D****E****F****G****H****I****J****K**

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 S3B**;  $p = 0.28$ ). 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 S2A**), 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 S2B**). 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 3G-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.

#### < **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  $200 \times 10^3$  cells per day to recipients that were simultaneously mobilized with G-CSF (G;  $10 \mu\text{g}$  per day intraperitoneally for 3 days) or PBS (P) as a control ( $n = 16-30$  per group). **(B)** The absolute number of  $\text{Lin}^-\text{Sca-1}^+\text{c-Kit}^{\text{H}}$  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- $\gamma$  and TNF- $\alpha$  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  $200 \times 10^3$  cells per day to recipients, followed by G-CSF administration on subsequent days ( $10 \mu\text{g}$  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 housekeeping 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  $\pm$  SEM are depicted. \* $p < 0.05$  \*\* $p < 0.01$  \*\*\* $p < 0.001$ .

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.<sup>20</sup> However, these results are in line with previous observations.<sup>19</sup> 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).

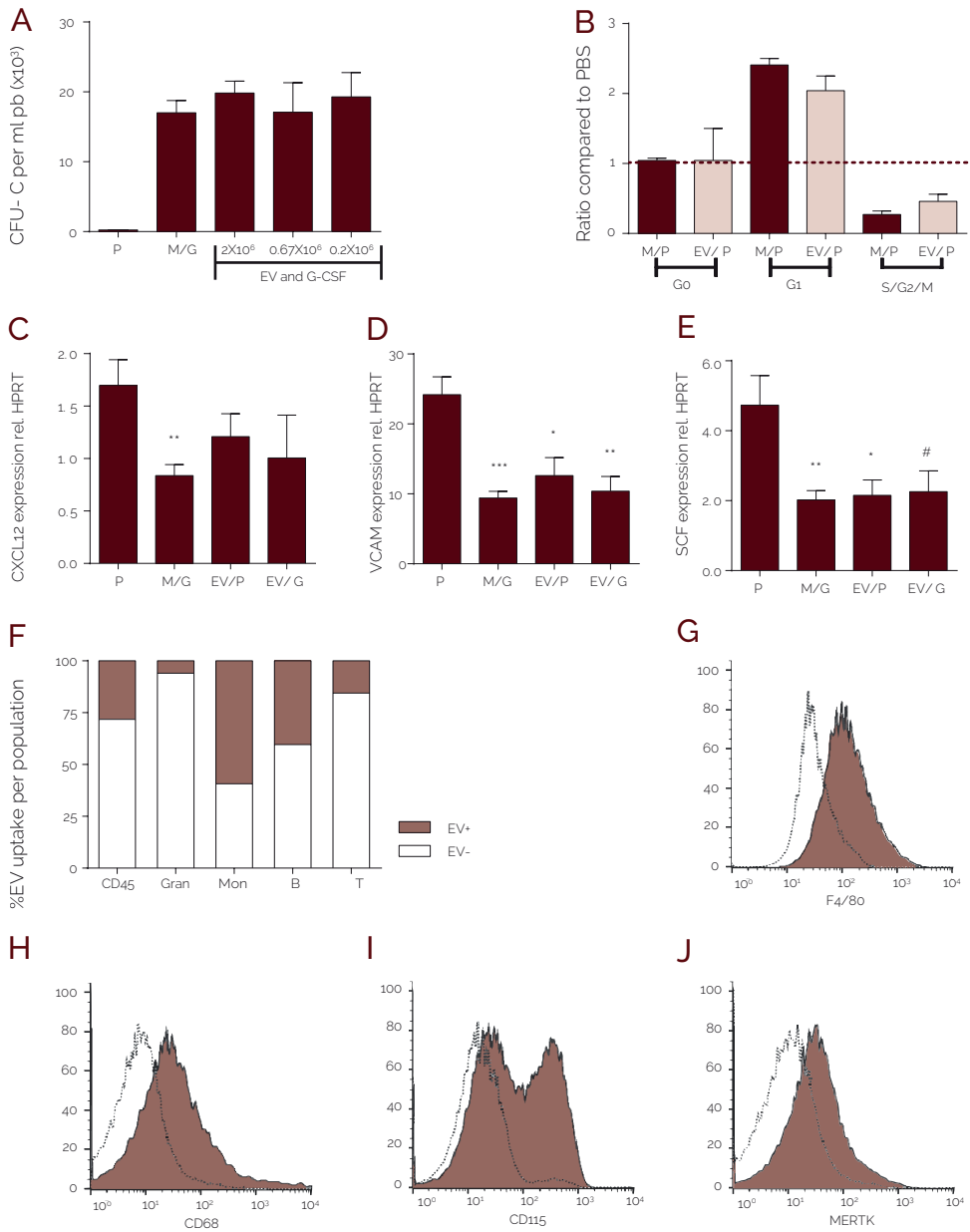
### MSC-DERIVED EXTRACELLULAR VESICLES ENHANCE G-CSF INDUCED HSPC MOBILIZATION

MSC have been reported to secrete extracellular vesicles (EV).<sup>27</sup> To investigate whether EV are the supernatant-derived factor that enhanced G-CSF-induced HSPC mobilization, EV that were derived from  $2 \times 10^6$ - $0.2 \times 10^6$  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 4B-E). Previously, it has been shown that MSC-derived EV migrate to the BM.<sup>28</sup> 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<sup>+</sup> cells were identified. About 28% of the CD45<sup>+</sup> 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<sup>POS</sup> monocytic cells. EV<sup>POS</sup> monocytic cells expressed F4/80, CD68 and MERTK at higher levels than EV<sup>NEG</sup> monocytic cells. In addition, about 50% of the EV<sup>POS</sup> cells expressed the M-CSF receptor (CD115; Figure 4G-J). This indicates that macrophages are the main EV-engulfing population in the BM.

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#### FIGURE 4. MSC-derived extracellular vesicles enhance G-CSF-induced mobilization. >

(A) MSC (M) or EV that were derived from  $2 \times 10^6$ - $0.2 \times 10^6$  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<sup>+</sup> 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<sup>neg</sup> cells are depicted as a dotted line, EV<sup>pos</sup> cells are depicted as filled histograms. Mean  $\pm$  SEM are depicted. \*p<0.05 \*\*p<0.01 \*\*\*p<0.001

## DISCUSSION

MSC are a cellular component of the HSC niche and play a major role in the maintenance of HSC in the BM.<sup>1,2</sup> In addition, in an experimental transplantation model, co-administration of MSC and HSPC has a beneficial effect on HSC engraftment and hematopoietic recovery.<sup>18,19</sup> 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.<sup>29</sup> Furthermore, depletion of osteal macrophages and a downregulation of *Cxcl12*, *Scf* and *Ang-1* mRNA is also observed during G-CSF-induced HSPC mobilization.<sup>15</sup> In steady state, macrophages regulate granulopoiesis and induce HSPC egress from the BM through circadian regulation of *Cxcl12* in stromal cells.<sup>30</sup> The decrease in *Cxcl12* expression and HSPC egress is preceded by the downregulation of Liver X receptor (LXR)-target gene downregulation in macrophages.<sup>30</sup> Depletion of BM macrophages results in downregulation of *Cxcl12*, *Vcam* and *Scf*, increased HSC proliferation and HSPC mobilization.<sup>13-15,31</sup> 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 strengthens 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 altered 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<sup>+</sup>MERTK<sup>+</sup>CD68<sup>+</sup> BM-derived macrophages. Previously, it has been shown that CD68<sup>+</sup> cells that express the G-CSF-receptor mediate G-CSF-induced HSPC mobilization.<sup>32</sup> 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.<sup>33–35</sup> MSC-derived EV are also thought to play a supporting role in tissue homeostasis and to influence responses to injury and infection.<sup>27,36</sup> EV, secreted by murine- or human MSC, are able to inhibit radiation-induced apoptosis of the murine hematopoietic cell line FDC-P1.<sup>28</sup> 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.<sup>37</sup>

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).

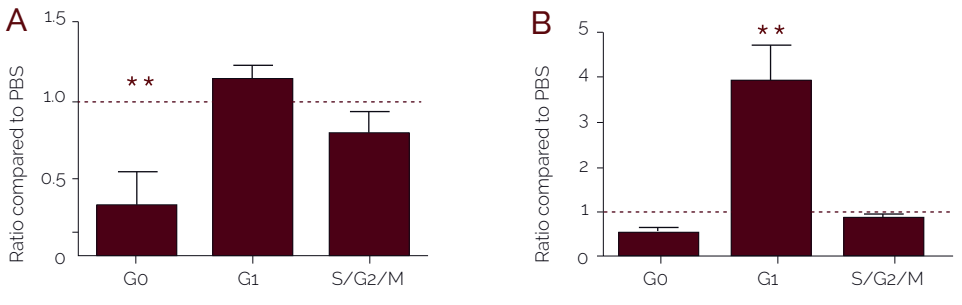
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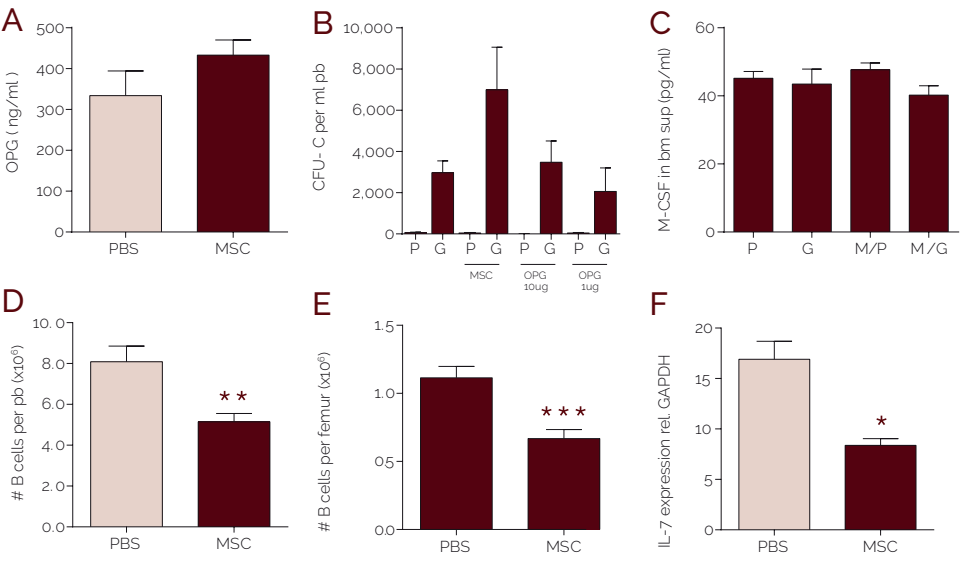
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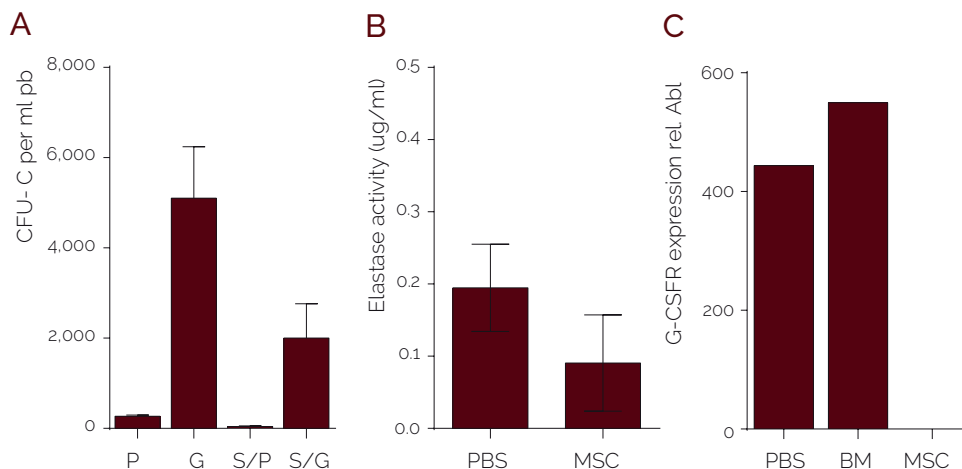
# SUPPLEMENTAL FIGURES



**S. FIGURE 1. MSC administration increases HSPC cell cycle activity.**  
Following 3 days of intravenous MSC administration, 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  $\pm$  SEM,  $n=7$  per group  $^{**}p<0.01$  compared to PBS.



**S. FIGURE 2. Effect of MSC administration on the hematopoietic stem cell micro-environment.**  
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  $\mu$ g or 1  $\mu$ g 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.  $^*p<0.05$ ,  $^{**}p<0.01$ ,  $^{***}p<0.001$



**S. FIGURE 3.**

**(A)** Splenocytes (S) were administered intravenously for 3 days at a dose of  $200 \times 10^3$  cells per day to recipients that were simultaneously mobilized with G-CSF (G; 10  $\mu$ g per day intraperitoneally for 3 days) or PBS (P) as a control (n= 3 per group). Data are depicted as mean  $\pm$  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  $\mu$ l cold PBS. The cell suspension was centrifuged at 2,300 g for 5 minutes and the supernatant was stored at -20  $^{\circ}$ 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  $\pm$  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).





# CHAPTER 6

## GENERAL DISCUSSION

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Adapted from: Cytokine-induced hematopoietic stem and progenitor cell mobilization: unraveling interactions between stem cells and their niche.

Published in: *Annals N Y Acad Science* 2019 (in press)

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The transplantation of hematopoietic stem and progenitor cells (HSPC) is a widely used procedure to treat malignant and non-malignant diseases of the blood and bone marrow (BM). Transplantation of HSPC in an autologous setting provides hematopoietic rescue after high-dose cytoreductive therapy, while transplantation in an allogeneic setting provides immune tolerance to donor cells, allowing donor T cells to mediate a graft-versus-tumor or graft-versus-leukemia effect. In addition, HSPC can be transplanted as a rescue therapy to treat immunodeficiency. HSPC that have been mobilized from the BM towards the peripheral blood by granulocyte colony-stimulating factor (G-CSF) have largely replaced BM-derived HSPC as a source for autologous stem cell transplantation and are currently used in the majority of allogeneic stem cell transplantations.<sup>1,2</sup>

The use of mobilized HSPC has several advantages over traditional BM-derived HSPC, for both donor and patient. The collection of peripheral blood HSPC through apheresis is a less invasive procedure than harvesting HSPC from BM and is associated with a decreased occurrence of adverse reactions in the donor. This results in a reduced time to recovery of HSPC donors after mobilization compared to donors undergoing BM aspiration.<sup>3</sup> Higher HSPC yields (expressed as CD34<sup>+</sup> cell dose) can be obtained through mobilization of HSPC as compared to direct harvest from the BM. It has been established that a minimum number of  $2.0 \times 10^6$  CD34<sup>+</sup> cells/kg body weight is required for a successful autologous transplantation.<sup>4</sup> Patients transplanted with mobilized HSPC generally receive a higher median number of HSPC and are more likely to maintain their graft in comparison to patients receiving BM-derived transplants.<sup>5</sup> The higher HSPC yield obtained through HSPC mobilization has allowed for the development of novel HSPC transplantation modalities, such as unrelated transplantation, haploidentical transplantation and non-myeloablative transplantation. For myeloablative and non-myeloablative allogeneic transplantation, a minimum threshold of  $3.0 \times 10^6$  CD34<sup>+</sup> cells/kg of body weight is commonly recommended. However, to improve engraftment and overcome rejection in haplotype mismatched transplantations, doses exceeding a threshold of  $10 \times 10^6$  CD34<sup>+</sup> cells/kg of body weight are needed.<sup>6</sup> As higher CD34<sup>+</sup> cell doses accelerate hematopoietic recovery, the transplantation of high numbers of CD34<sup>+</sup> cells is also important for transplantations in elderly patients, who have an increased risk of transplantation-related morbidity and mortality.<sup>7</sup>

Unfortunately, many donors are "poor mobilizers" as they fail to mobilize in response to G-CSF. Depending on the study population, this mobilization failure rate can be as high as 40 percent.<sup>4</sup> Several factors are associated with mobilization failure, including advanced age, a diagnosis of lymphoma, previous radiotherapy or extensive chemotherapy, treatment with immunomodulatory drugs or purine analogs, previous mobilization failure and low levels of pre-apheresis circulating peripheral blood CD34<sup>+</sup> cells.<sup>4</sup> Moreover, diabetes mellitus also correlates with a lower CD34<sup>+</sup>

yield after cytokine-induced HSPC mobilization.<sup>8</sup> This so-called "mobilopathy" is probably multifactorial; factors that may contribute to defective HSPC mobilization include microangiopathy, which results in quantitative and qualitative defects in BM microvasculature; sympathetic nervous system (SNS) dysfunction; an increase in BM adipocytes; and increased inflammatory macrophage numbers.<sup>9</sup>

However, it is difficult to predict mobilization failure in an individual donor, because even patients which lack high-risk characteristics may mobilize poorly in response to G-CSF.<sup>4</sup> It is therefore important to gain knowledge about the underlying mechanisms of HSPC mobilization in order to devise efficient strategies to obtain the maximum yield of mobilized HSPC from stem cell donors, as high numbers of transplanted HSPC accelerate hematopoietic recovery and reduce morbidity of the recipient.

After the initial discovery that endotoxin induces mobilization of HSPC from the BM towards the peripheral blood, many agents, including hematopoietic growth factors, chemokines and other molecules have been identified to induce HSPC mobilization.<sup>10</sup>

The mechanisms leading to HSPC mobilization have been studied extensively in the past decades, mainly through experiments in mice. These experiments, in combination with observations in humans, have led to the current understanding of the complex pathways and cellular components involved in HSPC mobilization.

In this chapter, an overview of key players in HSPC mobilization is provided, with emphasis on the role of hematopoietic and stromal cells. Furthermore, current and future mobilizing strategies will be discussed in relation to the data presented in this thesis.

## HEMATOPOIETIC CELLS IN HSPC MOBILIZATION

### NEUTROPHILS

As mentioned in **chapter 1** of this thesis, for many years, research in the field of HSPC mobilization focused on the role of neutrophils and neutrophil-derived proteases. Neutrophils play an essential role in HSPC mobilization induced by the cytokine interleukin-8 (IL-8) or by the chemokines GRO $\beta$ /CXCL2 and GRO $\beta$ T/CXCL24.<sup>11,12</sup> However, in G-CSF-induced HSPC mobilization, the role of neutrophils is not as clearly defined. This is illustrated by a transgenic mouse model in which the G-CSF-receptor (G-CSF-R) is only expressed on CD68<sup>+</sup> monocytic lineage cells and not on neutrophils. G-CSF-induced HSPC mobilization in these transgenic mice is not reduced, suggesting that G-CSF-R signaling in monocytic cells is sufficient to induce HSPC mobilization.<sup>13</sup> What remains unchallenged however, is that neutrophils, upon direct or indirect activation by G-CSF, release proteases such as neutrophil elastase (NE), cathepsin G (CG) and matrix metalloproteinase-9 (MMP-9). This results in a proteolytic bone marrow environment, in which proteases

degrade factors that anchor HSC in the niche.<sup>14</sup> Protease inhibitors such as serpin1 (or alpha-1-antitrypsin (AAT)) and serpin3 (or alpha-1-antichymotrypsin (ACT)) can inhibit NE and CG respectively.<sup>15</sup> Our group has shown that in mice, low-dose total body irradiation (0.5 Gy) results in significant inhibition of G-CSF- and IL-8-induced HSPC mobilization as a result of increased levels of AAT in the BM.<sup>16</sup> When human AAT is administered to mice, it almost completely blocks IL-8-induced HSPC mobilization.<sup>16</sup> This suggests that the balance between proteases and protease-inhibitors is a factor in determining either the retention or mobilization of HSPC. It could therefore be hypothesized that modulation of AAT levels in humans could be a novel strategy to induce HSPC mobilization. However, at the time of these experiments little was known about the role of proteases versus protease-inhibitors in G-CSF-induced HSPC mobilization in the human setting. Previously, research from our group had shown that, similar to observations in mice, the serum levels of MMP-9 and NE in healthy stem cell donors increase significantly after 3 to 5 days of G-CSF administration.<sup>17</sup> As is shown in **chapter 4**, AAT serum levels increase as well during G-CSF-induced HSPC mobilization.<sup>18</sup> Both serum levels of MMP and NE, as well as serum levels of AAT correlate positively with the extent of CD34<sup>+</sup> mobilization. If HSPC mobilization would be completely dependent on the balance between proteases such as NE, CG and MMP-9 on one side and protease-inhibitors such as AAT on the other side, the inhibition or depletion of AAT in humans should lead to a higher number of HSPC in the peripheral circulation during steady state. However, when we tested this in patients with AAT deficiency, the frequency of steady state peripheral blood HSPC was not significantly different from controls.<sup>18</sup> A possible explanation for this observation could be that other protease-inhibitors sufficiently compensated for the decreased AAT serum levels in these patients. Although the levels of two other abundantly expressed protease-inhibitors (alpha-2-macroglobulin and secretory leukocyte proteinase inhibitor) were not increased, this could not be excluded. Alternatively, other mechanisms that are not dependent on the balance between neutrophil-derived proteases and their inhibitors may contribute to retention and mobilization of HSPC. This is supported by the finding in more recent years that the BM contains several types of hematopoietic cells, other than neutrophils, that contribute to HSPC mobilization, such as macrophages, osteoclasts and erythrocytes. It is therefore unlikely that the observations on the role of proteases and protease-inhibitors in mice and humans will translate into clinical applications in cytokine-induced HSPC mobilization.

## MACROPHAGES

In recent years macrophages have been shown to play a crucial role in HSPC mobilization. The depletion of osteal macrophages (also called osteomacs) on the endosteal surface of osteoblasts, either through the administration of G-CSF

or upon administration of macrophage-depleting agents, is associated with the downregulation of *Scf*, *Cxcl12* and *Vcam1* gene expression and subsequent HSPC mobilization.<sup>19</sup> Similarly, the depletion of BM-resident CD169<sup>+</sup> macrophages leads to the selective downregulation of HSC retention genes (including *Cxcl12*, *Angpt1*, *Scf* and *Vcam1*) in Nes-GFP<sup>+</sup> MSC, resulting in reduced CXCL12 levels and concomitant HSPC mobilization.<sup>20</sup> In steady state conditions, the depletion of BM resident macrophages increases both hematopoietic stem cell (HSC) proliferation and the absolute number of quiescent HSC.<sup>21</sup> Furthermore, CD169<sup>+</sup> macrophages are essential for supporting erythropoiesis due to the fact that these macrophages are an integral part of erythroblastic islands, where a central macrophage is surrounded by erythroid precursors in varying stages of development.<sup>22</sup> The depletion of CD169<sup>+</sup> macrophages, as a consequence of the administration of G-CSF or fms-like tyrosine kinase 3 ligand (Flt3 ligand, FL) in mice, leads to a transient decrease in intramedullary erythropoiesis.<sup>23,24</sup> CD169<sup>+</sup> macrophages are also essential to the circadian fluctuation in circulating HSC. Upon clearance of aged (CD62<sup>lo</sup>/CXCR4<sup>hi</sup>) neutrophils by CD169<sup>+</sup> macrophages in the bone marrow, the size and function of the hematopoietic niche is reduced and the release of HSPC into the periphery enhanced.<sup>25</sup> Macrophages also play a role in HSPC mobilization induced by leukocyte cell-derived chemotaxin 2 (LECT2), as the LECT2 receptor (CD209a) is mainly expressed on macrophages and osteolineage cells.<sup>26</sup> As these results underscore the importance of macrophages in HSPC mobilization, more research is needed to delineate the exact nature of the macrophage subpopulations involved in HSPC mobilization.

## OSTEOCLASTS

There is controversy with respect to the role of osteoclasts during steady state HSC maintenance and HSPC mobilization. Osteoclasts, morphologically defined as large, multinucleated, hematopoietic-derived cells located adjacent to osteoblasts and osteocytes, are responsible for the dissolution and resorption of bone. Osteoclast inhibition, either through administration of the osteoclast inhibitor zoledronate or using transgenic mouse models, enhances G-CSF-induced HSPC mobilization and decreases *Cxcl12*, *Jagged-1* and *Scf* expression.<sup>19,27</sup> However, as we show in **chapter 5**, the administration of the osteoclast inhibitor osteopontegrin does not seem to affect G-CSF mobilization. Activation of osteoclasts, using receptor activator of nuclear factor kappa-B ligand (RANKL), also decreases CXCL12 levels in the BM and induces HSPC mobilization.<sup>28</sup> In contrast, several other studies have reported that osteoclasts are dispensable for HSC maintenance in adult mice.<sup>29–31</sup> Although the data seem to be conflicting, these studies may suggest that HSC numbers and HSPC mobilization are regulated by the level of osteoclast inhibition or activation.

## ERYTHROCYTES AND THE COMPLEMENT SYSTEM

Several studies have shown that the complement system contributes to the retention and mobilization of HSPC. In comparison to wild-type mice, G-CSF-induced mobilization is significantly increased in mice deficient in the complement factor C3 and the C3a receptor.<sup>32</sup> Additionally, mice treated with the C3a receptor antagonist SB 290157 show significantly accelerated and enhanced G-CSF-induced mobilization.<sup>32</sup> Furthermore, mice that are deficient in mannan-binding lectin (MBL) or its MBL-associated serine proteases (MASP-1 and -2), which can trigger the classical complement cascade, are poor mobilizers in response to G-CSF.<sup>33</sup> Interestingly, MBL-deficiency is seen in around 10% of humans, but it is yet unknown if this results in impaired HSPC mobilization.

The complement cascade is activated by administration of G-CSF, resulting in the formation of the membrane attack complex that lyses peripheral blood erythrocytes. Since erythrocytes are major reservoirs of the bioactive lipid sphingosine-1-phosphate (S1P), this hemolysis results in the massive release of S1P in the peripheral blood. As S1P acts as a potent chemoattractant of HSPC in a dose-dependent manner, the formation of this counter-gradient contributes to HSPC mobilization.<sup>34</sup> Hematopoietic stem and progenitor cells express the S1P receptor S1P1; the inhibition of the S1P/S1P1 axis significantly reduces the egress of steady state HSPC to the BM and diminishes G-CSF-induced HSPC mobilization, which demonstrates the important role of erythrocytes and S1P in HSPC mobilization.<sup>35</sup>

## STROMAL CELLS IN HSPC MOBILIZATION

### MESENCHYMAL STROMAL CELLS

Mesenchymal stromal cells (MSC) are an essential part of the HSC niche and support HSC maintenance and quiescence in the BM. As described in **chapter 1**, several types of BM-resident MSC, such as CAR cells, Nes-GFP<sup>+</sup> MSC and LEPR<sup>+</sup> pericytes, express high levels of HSC-supporting factors, such as CXCL12 and SCF. Perturbation of the niche, through manipulation of BM-resident MSC, has been shown to result in mobilization of HSPC. This is most obvious after the administration of G-CSF, which results in the decreased expression of HSC-supporting and HSC-anchoring factors by MSC.<sup>36</sup> The decreased expression of CXCL12, SCF and other HSC-supporting factors in the BM in turn leads to mobilization of HSPC from the BM towards the peripheral blood. MSC not only play a role in HSPC mobilization, but also in the BM engraftment of HSPC, as MSC that are co-transplanted with CD34<sup>+</sup> umbilical cord blood-derived HSPC, improve both HSC engraftment and hematopoietic recovery.<sup>37,38</sup>

Given this important role of MSC in the BM niche and their effect on HSC

engraftment and mobilization, we investigated the effect of MSC administration on the hematopoietic BM compartment. In **chapter 5** we show that administration of bone-derived MSC in mice results in a reduction of the number of BM LSK cells and increased HSPC cell cycle activity. Furthermore, the number of BM macrophages is significantly reduced and BM niche factors including *Cxcl12*, *Scf* and *Vcam* were downregulated in endosteal cells.<sup>39</sup> Interestingly, even though the observed phenomena are similar to those observed after the administration of G-CSF, the administration of MSC does not result in HSPC mobilization in these mice. However, the combined administration of G-CSF and MSC leads to a twofold increase in HSPC mobilization as compared to G-CSF alone. This synergistic effect of MSC on G-CSF-induced HSPC mobilization is mediated by a soluble factor, as two crucial experiments show: first, intravenously injected MSC lodge in the pulmonary vasculature, as is shown using bioluminescence *in vivo* and second, MSC culture supernatant was shown to exhibit the same effects on HSPC mobilization as MSC injected as a cell suspension. Finally, we could show that extracellular vesicles (EV), secreted by MSC, are responsible for the observed effects, as administration of MSC-derived EVs recapitulated all the effects that were observed following MSC administration. How MSC-derived EVs exert their effects on the BM stem cell niche is a subject of further research. *In vitro* we show that F4/80<sup>+</sup>MERTK<sup>+</sup>CD68<sup>+</sup> BM-derived macrophages engulf MSC-derived EVs. It could be speculated that *in vivo* engulfment of these EVs affects BM macrophages, resulting in downregulation of HSC-supporting factors and subsequent enhanced HSPC mobilization upon G-CSF administration. As EVs contain many factors it would be very interesting to determine which component(s) is/are responsible for the observed effects. If the addition of this component to G-CSF would lead to a twofold increase in HSPC mobilization in humans as well, without any deleterious effects, this would have a significant clinical impact. Obviously, further research in preclinical models is needed before application in a human setting could be explored.

## OSTEOLINEAGE CELLS

The BM is encapsulated by highly mineralized bone, produced by osteolineage cells. These cells go through various differentiation stages, ultimately forming mature osteoblasts and osteocytes. Osteoblasts form a layer of bone-generating cells, the endosteum, and differentiate into osteocytes when embedded in the mineralized compact bone matrix. The role of osteolineage cells in HSPC mobilization is limited.<sup>40-44</sup> Manipulation of osteoblast numbers does not result in HSPC mobilization into the blood.<sup>43,44</sup> Furthermore, animal models have indicated that conditional deletion of *Cxcl12* or *Scf* from mature osteoblasts does not result in HSC mobilization.<sup>40-42</sup> Administration of G-CSF results in depletion of osteoblasts at the endosteal surface, probably as a result of osteomac depletion. This co-incides

with a reduction in the number of pre-pro-B, pro-B, pre-B and surface IgM<sup>+</sup> mature B cells in the BM, due to increased B cell apoptosis.<sup>45</sup> Medullary B lymphopoiesis is dependent on cell-cell contact between B cell progenitors and osteoblasts, as well as the presence of osteoblast-derived CXCL12 and IL-7. This suggests that inhibition of medullary B lymphopoiesis may be the result of G-CSF-induced osteoblast depletion.<sup>45,46</sup> Interestingly, as we show in **chapter 5**, administration of MSC to mice also results in depletion of osteomacs, downregulation of *IL-7* in bone-lining cells and a reduction in B lymphopoiesis.

The role of osteocytes in HSPC mobilization is still unclear: targeted ablation of osteocytes in a transgenic mouse model results in the failure to mobilize HSPC in response to G-CSF. However, the validity of this model was questioned as not only osteolineage cells, but also CXCL12-abundant reticular (CAR) cells were targeted in this model.<sup>47,48</sup>

Bone contains a high concentration of calcium ions at the HSC-enriched endosteal surface. HSC express the seven-transmembrane-spanning calcium-sensing receptor (CaSR) and thus respond to extracellular ionic calcium concentrations.<sup>49</sup> Experiments with CaSR-deficient mice suggest that the CaSR retains HSC at the BM endosteal surface and the absence of CaSR on HSC impairs stem cell engraftment.<sup>49</sup> However, a role of the CaSR in HSPC mobilization has not been identified.

## ENDOTHELIAL CELLS

The exact role of endothelial cells (EC) in HSPC mobilization from the BM into the circulation is not fully understood. Vascular EC are the most important source of endogenous G-CSF, which plays a role in the body's response to physiological stress or bacterial infections.<sup>36</sup> Endothelial cells also express CXCL12, SCF and vascular cell adhesion molecule-1 (VCAM-1) on the cell surface, which are crucial HSC retention factors.<sup>40,41</sup> However, when *Cxcl12* is conditionally deleted from EC, HSC are depleted but not mobilized. This is likely related to the fact that the expression of CXCL12 is approximately 100-fold lower in EC in comparison to expression by perivascular MSC.<sup>40,50</sup> In the BM sinusoids, which are lined with EC, the transmembrane receptor for the ephrin B2 ligand (EPHB4), is widely expressed. Blockade of the interaction between EPHB4 and ephrin B2 on LSK cells reduces HSPC mobilization. This points towards a critical role for EPHB4/ephrin B2 signaling in mobilization of HSPC from the BM.<sup>51</sup>

## SYMPATHETIC NERVOUS SYSTEM

The role of the sympathetic nervous system (SNS) in HSPC maintenance under steady state conditions is well defined.<sup>52</sup> However, in cytokine-induced HSPC mobilization, its role is less apparent. The administration of G-CSF leads to increased sympathetic activity in the BM via impaired removal of noradrenaline

from the synaptic cleft.<sup>53</sup> Interestingly, sympathetic neurons express both G-CSF and G-CSF-R, where G-CSF likely plays a role as a protector against neurotoxic agents in an autocrine or paracrine fashion. Damage to the SNS due to neurotoxic chemotherapy, such as vincristine or cisplatin, results in impaired hematopoietic regeneration due to the selective loss of adrenergic innervation.<sup>54</sup> However, in mice treated with chemotherapy, adjuvant treatment with neuroprotective agents, such as 4-Methylcatechol or glial cell-derived neurotrophic factor, can rescue BM engraftment and mobilization.<sup>54,55</sup> Neuro-adrenergic stimulation can be used to increase HSPC mobilization, as was shown in a trial in which multiple myeloma patients were treated with a combination of G-CSF and the noradrenaline reuptake inhibitor desipramine.<sup>56</sup>

Sympathetic nerves also secrete neuropeptide Y (NPY), which is one of the most abundant and widely secreted peptides from the brain and SNS. In addition to its role in EC-regulated vascular permeability, NPY also induces HSPC mobilization through the Y1 receptor in osteoblasts by activating MMP-9.<sup>57</sup>

## CLINICAL APPLICATION OF MOBILIZING AGENTS

A wide variety of hematopoietic growth factors, chemokines, chemotherapeutic agents and other molecules that can induce HSPC mobilization have been identified since the first mobilization experiments using endotoxin. As described in **chapter 1**, G-CSF is still the most widely used mobilization agent, but several other agents have been approved for HSPC mobilization in a clinical setting, such as GM-CSF, SCF and plerixafor or AMD3100. Other agents, such as IL-8, FL, VCAM-1/VLA-4 inhibitors and S1P agonists, are mainly used in experimental animal studies or have been tested in early phase trials in human patients.<sup>1</sup>

## GRANULOCYTE COLONY-STIMULATING FACTOR

Although G-CSF has been used as a mobilizing agent for over 25 years, the multifaceted and interconnected mechanisms by which G-CSF induces HSPC mobilization have only come to light in the past few years.<sup>58</sup>

When G-CSF is administered, the number of neutrophils in the BM expands. Through increased reactive oxygen species (ROS) production, neutrophil expansion in response to G-CSF is associated with suppression of osteolineage cell populations in the BM, resulting in MSC and osteoblast apoptosis.<sup>59</sup> Furthermore, neutrophil activation initiates the release of proteolytic enzymes that cleave and inactivate chemokines and adhesion factors, such as CXCL12, SCF and VCAM-1 (**Figure 1**).<sup>60</sup> Administration of G-CSF also activates the complement cascade, resulting in release of C5a. The interaction of C5a with its receptor expressed on granulocytes subsequently activates phospholipase C- $\beta$ 2 (PLC- $\beta$ 2).

This, in turn, disrupts HSPC membrane lipid rafts containing adhesion molecules such as VLA-4 and CXCR4.<sup>61</sup> In addition, G-CSF depletes osteoblast-supportive endosteal macrophages and CD169<sup>+</sup> macrophages, inducing osteoblast ablation and blocking bone formation.<sup>19,62–64</sup> Together, this results in the reduced expression of chemokines and cytokines such as CXCL12, SCF and angiopoietin-1, which are necessary in order to maintain and retain HSC in their BM niches.<sup>63</sup> Osteoblast ablation might also result in a decrease in intramedullary AAT and ACT, thus promoting a proteolytic environment and amplifying the effects of G-CSF.<sup>16,65</sup> Administration of exogenous G-CSF drives BM-resident HSC into cell cycle, leading to increased HSC numbers in the BM, whereas G-CSF-mobilized peripheral blood

**A**

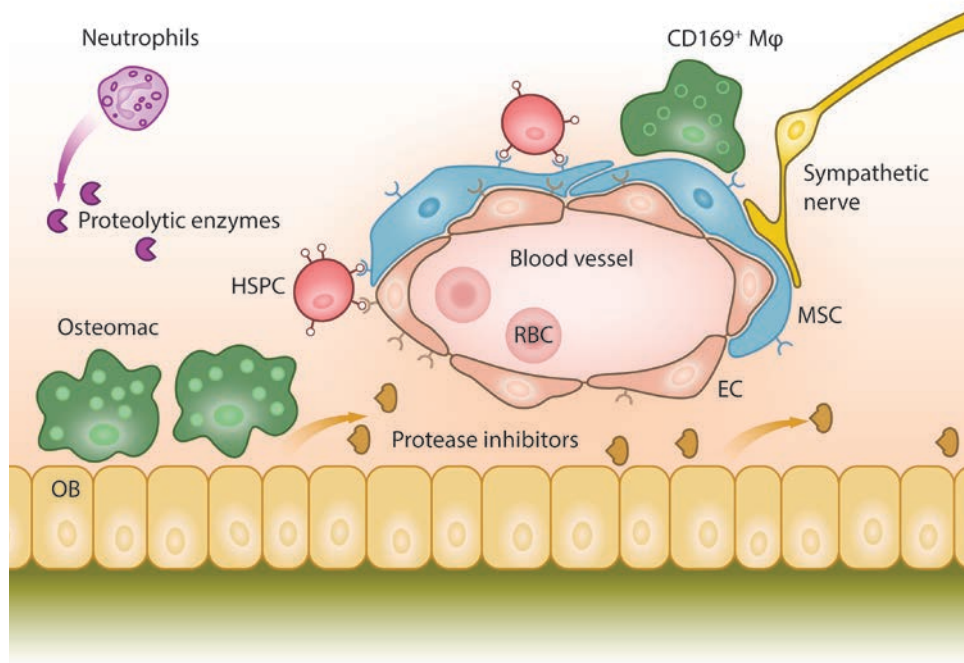


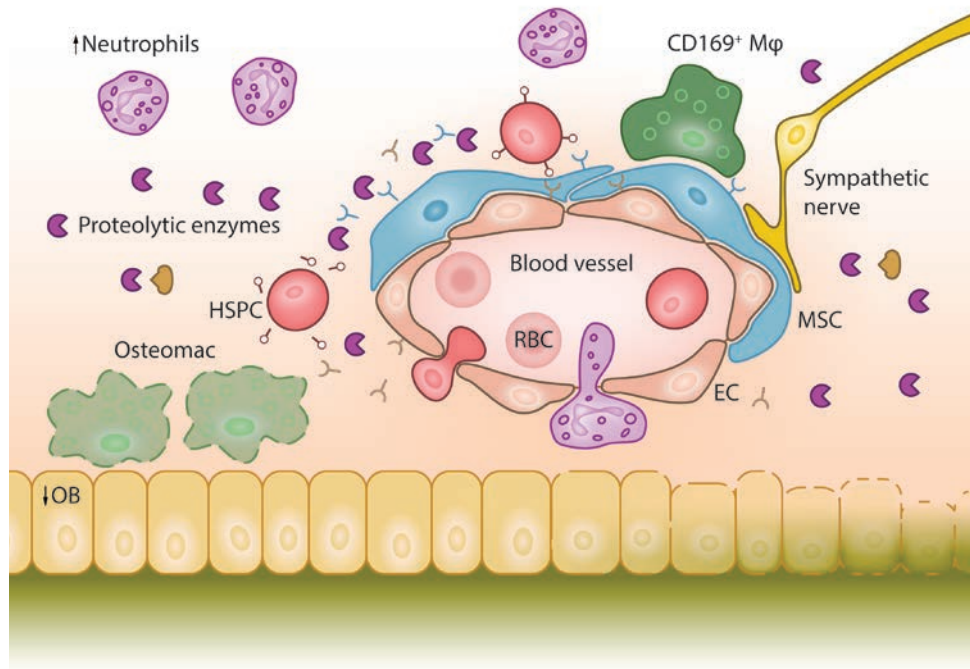
**FIGURE 1.** The BM niche in steady state and during G-CSF-induced HSPC mobilization.

**(A)** Steady state. Mesenchymal stromal cells (MSC) and endothelial cells (EC) express chemokine and adhesion molecules that retain hematopoietic stem and progenitor cells (HSPC) in the BM niche. Osteoblasts (OB) secrete protease-inhibitors that inhibit the proteolytic activity of neutrophil-derived proteases. Osteoblast-supportive endosteal macrophages (osteomacs) form a canopy over the bone-lining osteoblasts; CD169<sup>+</sup> macrophages (CD169<sup>+</sup> Mφ) support the stromal cells in the niche. RBC: red blood cell.

HSC are predominantly in the G0 or G1 phase.<sup>66</sup> It was recently shown that G-CSF does not uniformly mobilize HSC and HPC according to their maturation stage, but instead that the most potent HSC (defined as HSC able to serially transplant and reconstitute recipients) are mobilized as early as day 2 of G-CSF treatment.<sup>67</sup>

It is obvious that the effects of G-CSF administration on the BM niche are diverse and often seem deleterious to certain cell populations involved. Although studies on the long-term effects of G-CSF administration in healthy donors do not point towards significant permanent risks, not much is known about the reversibility of many of the short-term effects of G-CSF administration. Hypothetically, repeated G-CSF administration might induce permanent damage to the BM HSC niche,

**B**



**(B)** G-CSF-induced mobilization. Following G-CSF administration neutrophils in the BM expand, initiating the release of proteolytic enzymes that cleave and inactivate chemokines and adhesion factors, such as CXCL12, SCF and VCAM-1. Osteomacs are depleted, coinciding with osteoblast depletion and reduced secretion of protease-inhibitors, such as alpha-1-antitrypsin. This is associated with decreased expression of CXCL12, SCF and VCAM-1, which are required to maintain and retain HSPC in their BM niches. Increased sympathetic nerve activity leads to downregulation of CXCL12, SCF and VCAM-1 by stromal cells. Together these processes result in HSPC mobilization towards the peripheral blood.

which would translate in decreased mobilizing capacity. Furthermore, if only a fraction of mobilized HSPC homes towards the BM after they have been mobilized, repeated G-CSF administration might lead to exhaustion of the stem cell pool. In **chapter 3** we addressed these questions by administration of repeated cycles of recombinant-human-G-CSF as well as recombinant-murine-G-CSF in mice.<sup>68</sup> After 4 repeated cycles of recombinant-human-G-CSF, HSPC mobilization was decreased, with a further reduction in HSPC mobilization to baseline levels from 6 to 12 cycles onwards. Further analysis indicated that this was caused by the development of murine, neutralizing anti-recombinant-human-G-CSF antibodies. When the same experiments were repeated with recombinant-murine-G-CSF, which shares a 72,8% homology with recombinant-human-G-CSF, no inhibitory effects on HSPC mobilization were observed. In addition, BM neutrophil numbers, mobilizing capacity after IL-8 administration and the BM HSPC pool also remained unaffected. At the time of these experiments, the BM HSC niche was merely a concept and not amenable to comprehensive analysis. However, it would be of interest to study the HSC niche and its constituents in more depth under the conditions of the experiments mentioned. This would allow for the observation of possible permanent qualitative or quantitative changes in the HSC niche following G-CSF administration to HSPC donors. Although administration of repeated series of G-CSF is not common practice for mobilization purposes, patients with severe congenital neutropenia or other types of acquired neutropenia are often subject to prolonged G-CSF administration.<sup>69</sup>

### AMD3100 AND OTHER CXCL12/CXCR4 AXIS ANTAGONISTS

Preclinical studies show the important role of the CXCL12/CXCR4-axis in cytokine-induced HSPC mobilization. As described in **chapter 1**, CXCL12 is constitutively produced at high levels by various stromal niche cells in the BM and plays an important role in the homing and retention of HSPC.<sup>70,71</sup> The conditional deletion of CXCR4 or its ligand CXCL12 results in increased HSPC numbers in the peripheral blood and spleen.<sup>71</sup> Intracellular signaling enzymes, such as Rac1 and Rac2, are activated in response to CXCL12-signalling in HSPC. The depletion of both Rac1 and Rac2 in a transgenic mouse model leads to a massive egress of HSPC into the blood from the BM.<sup>72</sup> Through phosphoproteomic profiling of murine HSPC, it was recently shown that other proteins, such as the Rac1 activation protein ARHGAP25, are involved in HSPC mobilization via the CXCL12/CXCR4 pathway.<sup>73</sup> *In vivo*, CXCL12 is truncated by the membrane-bound extracellular peptidase CD26 (DPP4/dipeptidyl peptidase-4), which is expressed on the surface of many cell types in the BM, amongst which EC and a subset of HPC.<sup>74,75</sup> This peptidase is essential for G-CSF-induced HSPC mobilization, as CD26<sup>-/-</sup> mice show significantly decreased G-CSF-induced HSPC mobilization compared to wild-type mice.<sup>76</sup> This is most

likely due to altered CD26-dependent NPY signaling in sinusoidal EC, resulting in increased vascular permeability and subsequent HSPC egress.<sup>75</sup> Experiments in rats suggest that, in diabetes mellitus, reduced CD26 activity in the BM contributes to impaired HSPC mobilization in response to G-CSF.<sup>77</sup>

In mice it was shown that, through the downregulation of CXCR4 on the surface of HSPC and the alteration of the plasma-to-marrow CXCL12 gradient, the CXCR4 agonist peptide CTCE-0021 rapidly mobilizes neutrophils and HSPC to the peripheral blood.<sup>78</sup> This peptide works synergistically with G-CSF, resulting in a more than five-fold increase in HSPC mobilization.<sup>78</sup>

Following these preclinical studies, several drugs that target the CXCL12/CXCR4 axis have been approved for clinical use in humans or are in early phase trials. AMD3100 is a small-molecule bicyclam drug, that selectively interrupts the CXCL12/CXCR4 axis, with no inhibitory effect on other chemokine receptors.<sup>79</sup> Since CXCR4 serves as a co-receptor for the human immunodeficiency virus (HIV) to enter the cell, AMD3100 was initially developed as an anti-HIV drug. During phase I clinical trials in human volunteers, where AMD3100 was evaluated for its antiviral activity, it was noted that there was a rapid increase in peripheral white blood cells, peaking 6 hours after intravenous administration.<sup>80</sup> Subsequent studies have shown that a single injection of AMD3100 mobilizes CD34<sup>+</sup> HSPC to the peripheral blood.<sup>81</sup> Furthermore, administration in conjunction with G-CSF results in a tripling of circulating CD34<sup>+</sup> HSPC in comparison to either single agent G-CSF or AMD3100.<sup>82</sup> Based on two phase III clinical trials, AMD3100 is currently approved in the US and Europe for clinical HSPC mobilization in combination with G-CSF for patients with multiple myeloma or non-Hodgkin lymphoma that have failed to mobilize in response to G-CSF alone.<sup>83,84</sup> A recent study investigated the combination of AMD3100 and the CXCR2 agonist GRO $\beta$  in mice, showing rapid HSPC mobilization after a single co-injection of GRO $\beta$  and AMD3100, which was equivalent to 5 days of G-CSF administration.<sup>85</sup> Combined administration of GRO $\beta$  and AMD3100 resulted in the mobilization of HSPCs with increased engraftment potential compared to G-CSF-mobilized HSPC.

Further studies in humans are needed to evaluate the feasibility of this combination in clinical practice.

### FLT3 LIGAND

As described in **chapter 1** of this thesis, the cytokine FL, either alone or in combination with other growth factors, stimulates the proliferation of highly enriched human and murine HSC *in vitro*; *in vivo* FL leads to the expansion and mobilization of HSPC in animals and humans.<sup>86-88</sup> Unlike AMD3100 and G-CSF, FL is a 'slow mobilizer', i.e. it takes up to 10 days before treatment with FL results in the mobilization of HSC. This provides a time frame to study the kinetics of HPC and HSC mobilization on a day by day basis. In **chapter 2** we describe how FL

administration in mice results in differential mobilization of HPC and HSC. HPC are mobilized from day 3 of FL administration onwards, whereas significant numbers of HSC (defined as cobblestone area-forming cell (CAFC)-week 4 and -week 5) were only detected after 10 days of FL administration. Peripheral blood mononuclear cells (PBMC) obtained from mice treated with FL for 5 days were not radioprotective *in vivo*, whereas PBMC obtained from mice treated with FL for 10 days, showed 100% radioprotection. This is in marked contrast to the recent observation that after G-CSF administration the most potent HSC are mobilized as early as day 2 of G-CSF treatment.<sup>67</sup> A possible explanation for this difference in mobilization kinetics could be that in FL HSPC only mobilize after several days of HSPC expansion within the bone marrow, whereas G-CSF might be able to rapidly mobilize HSC with serially repopulating capacity because of their perivascular localization.

As FL is a slow mobilizing agent, it is of interest to study the combination of FL with other mobilizing agents. In mice we combined FL with IL-8, which is a very fast (i.e. < 30 minutes) HSPC mobilizer. Co-administration of FL for 3-5 days with a single injection of IL-8 leads to a significant increase in the mobilization of radioprotective HSPC. Since IL-8 is not registered for use in a human setting, from a clinical standpoint it is more relevant to study the combination of FL with agents such as G-CSF and AMD3100. Interestingly, the administration of FL in combination with G-CSF, GM-CSF or AMD3100 also leads to significantly increased HSPC mobilization, with the combination of FL and AMD3100 being the most potent.<sup>88,89</sup> In humans, FL (termed CDX-301) is well tolerated and able to mobilize sufficient HSPC for transplantation following 10 days of daily injections.<sup>90</sup> So far, there is no clinically approved FL product, and more research is needed to warrant the clinical application of FL as monotherapy or in combination with AMD3100 or G-CSF as a mobilizing agent in humans.

## NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

Prostaglandin E2 (PGE2) is an endogenous lipid, produced by cyclooxygenase-2 (COX-2), which enhances HSC homing, survival and proliferation.<sup>91</sup> Treatment with NSAIDs, like the COX-1 and COX-2 inhibitor meloxicam, reduces PGE2 production and is associated with significant HSPC egress from the BM.<sup>92</sup> PGE2-receptor knockout mice show an increased number of peripheral blood HSPC, which is caused by reduced E-prostanoid (EP4) receptor signaling.<sup>92</sup> NSAID-induced HSPC mobilization is independent of the CXCL12/CXCR4-axis, but is associated with attenuation of osteolineage cells and a significant reduction in osteopontin, which acts as a niche retention factor.<sup>92</sup>

Based on these preclinical data, multiple myeloma patients received meloxicam in combination with G-CSF as a mobilization regimen. Patients receiving G-CSF and meloxicam showed increased HSPC mobilization compared to administration

of G-CSF alone. This resulted in fewer patients requiring more than 1 day of stem cell collection and a reduced need for plerixafor administration.<sup>93</sup> Hematologic recovery after transplantation and survival rates were similar between the two groups. Furthermore, treatment with meloxicam was well tolerated, making this a promising, supportive strategy for HSPC mobilization.<sup>93</sup>

## INTEGRIN ANTAGONISTS

Integrins such as leukocyte function-associated antigen-1 (LFA-1,  $\alpha$ L $\beta$ 2 integrin, CD11a/CD18), very late antigen-4 (VLA-4,  $\alpha$ 4 $\beta$ 1-integrin) and very late antigen-5 (VLA-5,  $\alpha$ 5 $\beta$ 1-integrin) are not only involved in the engraftment of HSC in mice and humans, but play an important role in HSPC retention and mobilization from the BM towards the peripheral blood. In mice and primates, blocking of the receptor-ligand interaction by neutralizing anti-VLA-4 or anti-VCAM-1 monoclonal antibodies results in significant HSPC mobilization.<sup>94,95</sup> The treatment of patients with natalizumab, a recombinant humanized monoclonal antibody against the  $\alpha$ 4 subunit of VLA-4, which is approved for the treatment of multiple sclerosis and Crohn's disease, results in the mobilization of HSPC in these patients.<sup>96</sup> However, the association of natalizumab with progressive multifocal leukoencephalopathy precluded its further clinical application. Other  $\alpha$ 4 antagonists, such as the orally bioavailable drug frigategrast, are being developed, but are not yet commercially available.<sup>97</sup>

The development of integrin antagonists for blocking the  $\alpha$ 9 $\beta$ 1 integrin, whose expression is restricted to HSPC, is promising. The small molecule N-(benzenesulfonyl)-L-prolyl-L-O-(1-pyrrolidinylcarbonyl)tyrosine (BOP), which is a dual  $\alpha$ 9 $\beta$ 1/ $\alpha$ 4 $\beta$ 1 integrin antagonist, mobilizes HSPC after a single dose in mice.<sup>98</sup> Administration of a single dose of BOP in combination with a single dose of AMD3100 mobilizes similar numbers of HSPC as is observed after 4 days of G-CSF. However, in comparison with G-CSF, the combination of BOP and AMD3100 results in significantly enhanced short- and long-term engraftment in mice, indicating that this combination may be a rapid and effective alternative to G-CSF.<sup>98</sup>

Combinations of these integrin- and CXCR4 antagonists have the potential to develop into an effective, single-dose, one-day strategy to mobilize HSPC in different clinical settings.

## SUMMARY AND FUTURE DIRECTIONS

HSPC mobilization involves a multifaceted and complex interaction of HSPC, stromal and hematopoietic niche cells as well as an array of cytokines, chemokines and small molecules.

The field of stem cell mobilization research has advanced immensely over the past decade. Major steps in the elucidation of the complex mechanisms of HSPC mobilization have been made. Understanding the underlying mechanisms of HSPC maintenance and mobilization has led to a plethora of agents with mobilizing capacity. However, except for AMD3100, only few of these agents have reached the stage of clinical application, and so far, G-CSF remains the backbone of HSPC mobilization in humans.

G-CSF has its own limitations, such as the necessity for prolonged parenteral administration and suboptimal efficiency in certain patient groups. Furthermore, although the administration of G-CSF is generally safe and serious adverse events are rare, bone pain and fatigue are experienced by a majority of donors and patients treated with G-CSF.<sup>3</sup> Therefore, there is an unmet need for innovative mobilizing agents or strategies. The identification of agents that are able to collectively influence the many mechanisms that underlie HSPC mobilization may provide substantial improvements to existing HSPC mobilization methods and subsequent transplant outcomes.<sup>99</sup> Ideally, these agents are potent HSPC mobilizers that can be titrated to the required peripheral blood HSPC dose, have an excellent safety profile, can be administered as a single dose and are not expensive.

Despite all efforts to elucidate the mechanisms underlying HSPC mobilization, there are still questions that require an answer before HSPC mobilization can be fully understood and manipulated.<sup>100</sup> These questions include:

- is the continuous exit of HSPC into the bloodstream in steady state regulated by the same mechanisms as cytokine-induced HSPC mobilization?
- what is the relative contribution of each cell population (e.g. macrophages, MSC) and their respective interactions and signals in cytokine-induced HSPC mobilization?
- can biomarkers be identified that predict the mobilizing capacity in response to mobilizing agents?

These questions, and likely many others, can drive future research and hopefully lead to better, safer and more efficient mobilization strategies.

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# CHAPTER 7

SUMMARY / NEDERLANDSE SAMENVATTING

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## SUMMARY

In humans and vertebrate animals, blood cells are produced in the bone marrow (BM). All blood cells are progeny of hematopoietic stem cells (HSC), which reside at the top of the hematopoietic hierarchy and give rise to committed hematopoietic progenitor cells (HPC). These HPC subsequently differentiate into lineage-restricted progenitors, including B cells, T cells, monocytes and granulocytes, with limited or no proliferative potential. Hematopoietic stem and progenitor cells (HSPC) reside in specific niches in the BM, where they are part of a complex micro-environment, comprising different subsets of hematopoietic and non-hematopoietic cells, as is described in detail in [chapter 1](#).

In a clinical setting, HSPC can be transplanted from donor to recipient to treat malignant as well as non-malignant diseases. HSPC can be obtained from a donor by two methods: harvesting directly from the BM through BM puncture or harvesting from the peripheral blood after the HSPC have migrated from the BM to the peripheral blood, a process called mobilization. The use of mobilized HSPC has largely replaced BM-derived HSPC as a source for both autologous and allogeneic transplantation, mainly because of its advantages such as less invasive harvesting procedures, higher HSPC yields and faster hematopoietic reconstitution after transplantation.

Under steady state conditions, the peripheral blood contains only a very small number of HSPC. Administration of several exogenous cytokines, chemokines, small molecules and chemotherapeutic agents mobilizes HSPC from the BM. Administration of a single growth factor, granulocyte-colony-stimulating factor (G-CSF), has become the standard regimen for HSPC mobilization in humans. However, the events that lead to G-CSF-induced HSPC mobilization are yet incompletely understood. This is problematic, since a significant proportion (of up to 40%) of stem cell donors fail to mobilize in response to G-CSF. Therefore, understanding the sequence of events leading to HSPC mobilization is fundamental to develop novel strategies and to optimize current mobilization protocols. This thesis focuses on the mechanisms that underlie HSPC mobilization, using both mouse models and observational studies in humans.

### KINETICS OF HSC AND HPC MOBILIZATION

In [chapter 2](#), the use of the cytokine Flt3-ligand (FL) as a model to study the kinetics of HSC and HPC mobilization is described. FL is a slow mobilizing agent, as it takes 5-10 days to mobilize HSPC from the BM to the blood, whereas G-CSF takes 3-5 days for its maximum effect. This slow mobilization process provides a time-frame to study HSPC migration kinetics in detail. HPC, which are short-term reconstituting cells, were measured by determining colony-forming units-

granulocytes/macrophages (CFU-GM) colony formation. Following intraperitoneal administration of FL to mice for 3-10 days, CFU-GM increase significantly from day 3 onwards, showing the mobilization of HPC. HSC, that have long-term repopulating capacity, were measured by cobblestone-area-forming cell (CAFC) assay at day 28 and day 35. CAFC-day 28 and -35 was significantly higher after administration of FL for 10 days then after FL administration for 5 days. These results were corroborated by radioprotection assays where no radioprotection was obtained after transplantation of 5-day FL-mobilized peripheral blood mononuclear cells (PBMC), whereas 10-day FL-mobilized PBMC radioprotected 100% of the recipients with significant (> 90%) multilineage donor chimerism. This indicates that HPC and HSC show different mobilization kinetics in response to FL, resulting in preferential mobilization of HPC at day 5, followed by HSC mobilization at day 10. Interestingly, Flt3 is not expressed on HSC, thus mobilization of HSC by FL can only be induced through an indirect mechanism, such as disruption of the CXCL12/CXCR4-axis or by expansion of neutrophils in the BM, which in turn promotes HSC mobilization through the release of proteases. When FL was given together with IL-8, synergistic enhancement of HSC and HPC mobilization on day 3 and 5 was seen, as compared to administration of FL or IL-8 alone. Superior overall survival in a radioprotection assay was seen in the mice receiving both FL-5 days and IL-8, which may be explained by the higher number of HPC with short term radioprotection capacity that are necessary for the early survival after lethal total body irradiation. This, in turn, allows a time frame for the engraftment of HSC that sustain long term survival of the recipient mice.

## REPEATED G-CSF-INDUCED HSPC MOBILIZATION

When G-CSF is used as a mobilizing agent, peripheral blood HSPC numbers increase steadily and reach a plateau between day 5 and 10 of G-CSF administration, after which they decrease. Experiments in parabiotic mice suggest that mobilized HSPC can return to the BM. However, a proportion of mobilized HSPC might not home back to their BM niches and are triggered to enter apoptosis. If this would be the fate of a significant amount of mobilized HSPC, repeated cytokine-induced mobilization would lead to depletion of the BM stem cell pool and ultimately to BM hypoplasia and pancytopenia. In **chapter 3**, we describe experiments in which G-CSF was repeatedly administered for 5 days in two-week cycles to mice, for a total of 12 cycles. The administration of recombinant human G-CSF (rhG-CSF) lead to reduced peripheral blood HSPC mobilization from cycle 4 onwards, until no significant HSPC mobilization was detected after 12 cycles. BM CFU-GM numbers as well as BM neutrophils did not significantly differ from cycle to cycle. When a single injection of IL-8 was administered after the final dose of rhG-CSF, normal HSPC mobilization was observed, showing that the absence of HSPC mobilization

was not caused by a defect in mobilizing capacity. However, the observed reduction in HSPC mobilization in these mice was caused by neutralizing anti-rhG-CSF antibodies, that were found in the plasma of mice mobilized with 2 or more cycles of rhG-CSF, showing that the use of a xenogeneic cytokine can have limitations in a murine model of HSPC mobilization. In contrast, following repeated administration of recombinant murine G-CSF, HSPC mobilizing capacity remained intact for up to 12 cycles, suggesting that repeated administration of G-CSF does not lead to depletion of the BM HSPC pool.

## PROTEASE INHIBITION AND HSPC MOBILIZATION IN HUMANS

G-CSF-induced HSPC mobilization is associated with an increase in BM and peripheral blood neutrophils. Previous work from our group showed the important role of neutrophils in cytokine-induced HSPC mobilization. Upon the administration of G-CSF, proteases including neutrophil elastase (NE), cathepsin G (CG) and matrix-metalloproteinase-9 (MMP-9) are released from the granules of activated neutrophils. These proteases accumulate in the BM during HSPC mobilization, leading to a highly proteolytic environment. As a consequence, adhesive interactions which anchor HSPC in the BM niche, including VCAM-1/VLA-4 and CXCL12/CXCR4, are degraded, resulting in the mobilization of HSPC to the peripheral blood. Under physiologic conditions, the accumulation of high levels of proteolytically active proteases is strictly regulated by the presence of protease inhibitors. Protease inhibitors, such as serpin1 (or alpha-1-antitrypsin, AAT) and serpin3 (or alpha-1-antichymotrypsin, ACT), can inhibit the neutrophil-derived serine proteases NE and CG, respectively. In humans, the levels of proteases such as MMP-9 and NE increase in peripheral blood plasma after 3 to 5 days of daily G-CSF administration; these levels correlate with the extent of HSPC mobilization. In [chapter 4](#), the relationship between AAT and HSPC frequency in the peripheral blood in steady state and in cytokine-induced mobilization in humans is described. Patients with alpha-1-antitrypsin deficiency (AATD) have very low levels of peripheral blood AAT (10-15% compared to healthy individuals). We hypothesized that in these patients increased proteolytic activity in the BM would result in increased steady state peripheral blood HSPC numbers. However, using flow cytometry and semi-solid HPC culture, we did not observe significant differences in peripheral blood HSPC in AATD patients as compared to controls. This unexpected result could not be explained by a compensatory increase in the levels of other protease inhibitors, since levels of two other major protease inhibitors, alpha-2-macroglobulin and secretory leukoprotease inhibitor, were similar between the groups. This indicates that AAT is not an indispensable protease-inhibitor in the constitutive circulation of HSPC in humans.

Healthy stem cell donors were mobilized with G-CSF for 5 days and the number

of CD45<sup>+</sup>/CD34<sup>+</sup> HSPC was determined in the peripheral blood. During G-CSF-induced HSPC mobilization, peripheral blood AAT levels increased significantly, positively correlating with the extent of mobilized CD45<sup>+</sup>/CD34<sup>+</sup> cells. The increase in AAT levels is most likely a response to the high serum levels of proteases, such as NE, that is irreversibly bound by AAT. Although proteases play an important role in HSPC mobilization, subsequent studies in mice, deficient for one or more proteases, show HSPC mobilization in response to G-CSF. Taken together, these and our data show that both protease-dependent and -independent pathways contribute to the cytokine-induced egress of HSPC from the BM to the blood.

## HSPC MOBILIZATION AND THE BM NICHE

In recent years, it was shown that hematopoietic and non-hematopoietic cells that comprise the HSC niche, play an essential role in HSPC mobilization. Mesenchymal stromal cells (MSC) are a non-hematopoietic population of cells that have the capacity to differentiate into osteoblasts, adipocytes and chondrocytes. In the BM, MSC form part of the HSC niches surrounding arterioles and sinusoids. Given the key role of MSC in the HSC microenvironment and their effect on HSC engraftment and hematopoietic recovery, we performed experiments in mouse models, studying the effect of MSC administration on the hematopoietic BM compartment. In [chapter 5](#), we show that daily intravenous administration of MSC in mice resulted in increased HSC cell cycle activity in the BM. Furthermore, administration of MSC induced a significant decrease in endosteal macrophages and CD169<sup>+</sup> macrophages in the BM. HSC-retaining factors, such as *Cxcl12* and *Vcam* were significantly decreased in endosteal cells. Although the observed changes were similar to what is observed during G-CSF-induced HSPC mobilization, administration of MSC alone did not induce HSPC migration. However, co-injection of MSC and G-CSF induced a 2-fold increase in HSPC mobilization compared to G-CSF administration alone. In addition, co-administration of MSC and G-CSF downregulated the expression of *Cxcl12*, *Vcam* and *Scf* in endosteal cells more than G-CSF alone. Bioluminescence imaging of MSC revealed that intravenously injected MSC were trapped in the lungs and did not home to the BM. Co-injection of MSC culture supernatant and G-CSF resulted in similar enhancement of HSPC mobilization, therefore we hypothesized that MSC may exert their effect on the HSC niche through a soluble mediator. Since MSC secrete extracellular vesicles (EV), we investigated whether these EV are the supernatant-derived factor that enhanced G-CSF-induced HSPC mobilization. Indeed, co-administration of MSC-derived EV and G-CSF for 3 days induced HSPC mobilization at similar levels as co-injection of MSC and G-CSF, enhanced the cell cycle activity of HSPC and downregulated expression of *Cxcl12*, *Vcam* and *Scf* similar to MSC administration. Together these data indicate that, through the secretion of EV, administration of MSC induces a permissive state in the BM, in which

niche retention factors are decreased and HSPC are preferentially in cell cycle. This permissive state then allows for enhanced G-CSF-induced HSPC mobilization. Further research is needed to elucidate which factors that are contained by the EV are responsible for the observed phenomena.

Not only MSC and macrophages, but also other stromal and hematopoietic cells, such as erythrocytes, endothelial cells and sympathetic nervous system cells, form the BM HSC niche. The complex interactions between HSPC and these cell populations are described in **chapter 6**. A vast array of cytokines, chemokines and small molecules is involved in steady state hematopoiesis and in HSPC mobilization. Several of these agents, such as G-CSF, have an important role in the clinical application of HSPC mobilization in humans and their use is described in this chapter. Major steps in the elucidation of the complex mechanisms of HSPC mobilization have been made; however, there is still an unmet need for innovative mobilizing agents or strategies. Further research will hopefully address the remaining questions and lead to better, safer and more efficient mobilization strategies.

## NEDERLANDSE SAMENVATTING

Bij mensen en bij gewervelde dieren worden bloedcellen gemaakt in het beenmerg. Alle bloedcellen stammen af van zogeheten hematopoietische stamcellen (HSC), die aan de top van een hiërarchie van beenmerg- en bloedcellen staan. Deze stamcellen brengen weer voorlopercellen (HPC) voort, die op hun beurt differentiëren in bloedcellen die een specifieke functie hebben, zoals B-cellen, T-cellen, monocyt en granulocyten. Deze cellen hebben zelf de eigenschap om zich te delen verloren. Hematopoietische stam- en voorlopercellen (HSPC) verblijven in het BM in specifieke niches, waar ze onderdeel zijn van een complex micromilieu, bestaande uit verschillende soorten bloed-, beenmerg- en bindweefselcellen. In **hoofdstuk 1** wordt dit in detail beschreven.

In de klinische praktijk worden HSPC getransplanteerd van een donor naar een ontvanger, met als doel het behandelen van zowel kwaadaardige als goedaardige ziekten. HSPC kunnen op twee manieren van een donor worden verkregen: direct vanuit het beenmerg via een beenmergpunctie of via het bloed, volgend op migratie van HSPC vanuit het beenmerg naar het bloed. Dit proces van het bewegen van de HSPC van het beenmerg naar het bloed wordt "mobilisatie" genoemd. HSPC die verkregen zijn via mobilisatie worden tegenwoordig veel vaker gebruikt dan HSPC die via beenmergpunctie zijn verkregen, zowel voor autologe als voor allogene transplantatie. De reden hiervoor is dat er verschillende voordelen zijn aan het mobiliseren van HSPC: er is geen invasieve beenmergpunctie voor nodig, er kunnen meer HSPC worden verkregen per procedure en bij een patiënt die de cellen ontvangt treedt sneller herstel van de beenmergfunctie op.

Onder normale omstandigheden bevat het bloed slecht een klein aantal HSPC. De HSPC die zich in het beenmerg bevinden kunnen door een verschillend aantal stoffen worden gemobiliseerd naar het bloed, zoals cytokines, chemokines, bepaalde kleine moleculen of door chemotherapie. Toediening van een specifieke groeifactor, G-CSF (granulocyten-kolonie-stimulerende factor) is de standaardbehandeling voor het mobiliseren van stamcellen bij mensen. Echter, hoe dit precies in zijn werk gaat, is tot nu toe niet volledig bekend. Dat is een probleem, omdat (afhankelijk van bepaalde donorkarakteristieken) tot 40% van de stamceldonoren niet mobiliseert na toediening van G-CSF. Daarom is het van belang om de gebeurtenissen die leiden tot HSPC mobilisatie te begrijpen. Zo kunnen nieuwe strategieën worden ontworpen om het mobilisatieproces te optimaliseren.

Dit proefschrift richt zich op de mechanismen die ten grondslag liggen aan stamcelmobilisatie. Hierbij worden zowel muismodellen als waarnemingen bij patiënten en gezonde donoren gedaan.

## KINETIEK VAN HSC EN HPC MOBILISATIE

In **hoofdstuk 2** wordt het gebruik van het cytokine Flt3-ligand (FL) beschreven waarmee het mogelijk is om de kinetiek van de mobilisatie van HSC en HPC te bestuderen. FL induceert langzame mobilisatie omdat het 5 tot 10 dagen duurt om HSPC van het beenmerg naar het bloed te mobiliseren. Bij G-CSF duurt het 3-5 dagen voordat het gewenst effect bereikt wordt. Dit langzame mobilisatie proces geeft de gelegenheid om HSPC mobilisatie in detail te bestuderen. HPC zijn in staat om kortdurend het beenmerg te repopuleren. De hoeveelheid HPC in het bloed kan worden bepaald doordat zij in kweek kolonievormende units – granulocyten/macrofagen (CFU-GM) vormen. Als FL intraperitoneaal wordt toegediend bij muizen gedurende 3-10 dagen neemt het aantal CFU-GM significant toe vanaf dag 3, passend bij HPC mobilisatie. HSC hebben de capaciteit om te zorgen voor langdurige repopulatie van het beenmerg. Zij kunnen worden gemeten met behulp van een "cobblestone-area-forming cell" (CAFC) assay, indien deze wordt uitgelezen op dag 28 en op dag 35 na start van de kweek. CAFC dag 28 en dag 35 was significant hoger na toediening van FL gedurende 10 dagen dan na toediening gedurende 5 dagen. Deze resultaten werden bevestigd door bestralingsexperimenten bij muizen (middels een radioprotectie assay). Na transplantatie van bloedcellen die verkregen waren na 5 dagen FL werd geen radioprotectie gezien. Daarentegen beschermden bloedcellen verkregen bij muizen die gedurende 10 dagen met FL waren behandeld de muizen volledig. In deze groep muizen werd > 90% chimerisme gezien. Dit betekent dat HPC en HSC een verschillende kinetiek hebben na toediening van FL. Hierbij is er preferentiële mobilisatie van HPC op dag 5, gevolgd door HSC mobilisatie op dag 10. Flt3 is overigens niet aanwezig op HSC, zodat de mobilisatie van HSC door FL alleen door een indirect mechanisme kan zijn veroorzaakt. Een mogelijke verklaring is onderbreking van de CXCL12/CXCR4 as of toename van neutrofiele granulocyten in het beenmerg, waarbij proteases vrijkomen die HSC mobilisatie in gang kunnen zetten. Als FL gegeven werd in combinatie met IL-8 werd toegenomen mobilisatie van HSC en HPC op dag 3 en dag 5 na toediening gezien, vergeleken met FL of IL-8 alleen. Ook werd verbeterde overleving gezien in een radioprotectie assay indien FL gedurende 5 dagen gecombineerd werd met IL-8. Dit komt mogelijk door een hoger aantal gemobiliseerde HPC, die zorgen voor overleving in de vroege fase na stamceltransplantatie. Hierdoor wordt het mogelijk dat HSC zich in het beenmerg nestelen en langdurige overleving van de ontvangermuizen mogelijk maakt.

## HERHAALDE G-CSF TOEDIENING

Na toediening van G-CSF nemen de HSPC in het bloed geleidelijk toe. De gemobiliseerde HSPC bereiken een plateau tussen dag 5 en 10 na G-CSF toediening, waarna zij weer afnemen. Bij muizen waarbij op experimentele

wijze een gezamenlijke bloedsomloop bestaat, is aangetoond dat HSPC die gemobiliseerd zijn, ook weer kunnen terugkeren naar het beenmerg. Echter, een deel van de gemobiliseerde HSPC keert mogelijk niet terug naar hun beenmerg niche en gaat dood. Als dat voor een groot deel van de gemobiliseerde HSPC zou gelden, dan zou herhaalde G-CSF toediening kunnen leiden tot uitputting van de stamcelvoorraad en uiteindelijk tot beenmergfalen. In **hoofdstuk 3** worden experimenten beschreven, waarbij G-CSF in herhaalde cycli van 5 dagen werd toegediend in een tweewekelijks schema. In totaal werden maximaal 12 cycli van 5 dagen G-CSF toegediend. Bij toediening van recombinant humaan G-CSF werd verminderde HSPC mobilisatie vanaf cyclus 4 gezien. Na 12 cycli G-CSF werd in geheel geen mobilisatie meer waargenomen. In het beenmerg werd geen verschil gezien in het aantal CFU-GM en neutrofielen tussen de verschillende cycli. In een aanvullend experiment werd IL-8 toe gediend na de laatste injectie met G-CSF. Hierbij mobiliseerden de HSPC normaal, wat aantoonde dat er geen gestoorde mobilisatiecapaciteit was. Het bleek dat de afname in HSPC mobilisatie veroorzaakt werd door neutraliserende antilichamen gericht tegen recombinant humaan G-CSF. Deze konden worden aangetoond in het plasma van muizen die 2 of meer cycli recombinant humaan G-CSF hadden ontvangen. Dit toont aan dat het toedienen van een humaan cytokine zijn beperkingen kent bij toepassing in een muismodel voor HSPC mobilisatie.

Dat de afname in HSPC mobilisatie door deze antilichamen werd veroorzaakt, werd nog versterkt door de waarneming dat mobilisatie met recombinant muizen-G-CSF geen verminderde mobilisatie liet zien tot en met toediening van 12 cycli. Dit suggereert dat herhaalde toediening van G-CSF niet tot uitputting van de stamcelvoorraad leidt.

### PROTEASE REMMING EN HSPC MOBILISATIE BIJ PATIËNTEN EN DONOREN

G-CSF-geïnduceerde HSPC mobilisatie gaat samen met een toename van neutrofielen in beenmerg en bloed. Eerder onderzoek van onze groep toonde aan dat neutrofielen belangrijk zijn bij cytokine-geïnduceerde HSPC mobilisatie. Na toediening van G-CSF worden proteases losgemaakt uit korrels in het cytoplasma van de neutrofiel. Deze proteases zijn onder andere neutrofiel elastase (NE), cathepsine G (CG) en matrix-metalloproteinase-9 (MMP-9). Tijdens G-CSF mobilisatie neemt de hoeveelheid proteases in het beenmerg toe, waardoor een milieu ontstaat waarbij eiwitten makkelijk geknipt kunnen worden. Hierdoor worden verschillende verbindingen doorgeknipt, zoals VCAM-1/VLA-4 en CXCL12/CXCR4, die HSPC in het beenmerg verankeren. Dit resulteert vervolgens in mobilisatie van HSPC naar het bloed. Onder normale omstandigheden wordt door proteaseremmers voorkomen dat de hoeveelheid actieve proteases te hoog wordt. Proteaseremmers zoals serpina1 (alpha-1-antitrypsin, AAT) en serpina3 (alpha-1-

antichymotrypsin, ACT) kunnen de serine proteases NE en CG, die door neutrofielen worden uitgescheiden, remmen. Bij mensen neemt het niveau van de proteases MMP-9 en NE in het bloedplasma toe na 3 tot 5 dagen G-CSF toediening; dit niveau correleert met de mate van HSPC mobilisatie. In **hoofdstuk 4** wordt beschreven wat de relatie is tussen de hoeveelheid AAT en de HSPC frequentie onder normale omstandigheden en tijdens cytokine-geïnduceerde HSPC mobilisatie. Patiënten met alfa-1-antitrypsine deficiëntie hebben lage bloedspiegels van AAT (10-15% t.o.v. gezonde mensen). Wij hebben de hypothese getoetst dat bij deze patiënten, onder fysiologische omstandigheden, de toegenomen AAT activiteit in het beenmerg zou leiden tot een verhoogd aantal HSPC in het bloed. Door middel van flowcytometrie en HPC kweken kon dit echter niet worden aangetoond. Dit onverwachte resultaat kon niet worden veroorzaakt door een compensatoire toename van andere proteaseremmers, omdat de spiegels van de proteaseremmers alfa-2-macroglobuline en SLPI (secretory leukoproteïnase inhibitor) niet verschilden tussen de patiëntengroep en een groep gezonde vrijwilligers. Dit laat zien dat AAT geen onmisbare proteaseremmer is in de constitutionele circulatie van HSPC bij mensen. In een ander experiment werden gezonde stamceldonoren behandeld met G-CSF gedurende 5 dagen, waarbij het aantal CD45<sup>+</sup>/CD34<sup>+</sup> cellen (als maat voor HSPC) werd bepaald in het bloed. Gedurende G-CSF mobilisatie namen de AAT spiegels geleidelijk toe, evenredig met het aantal gemobiliseerde CD45<sup>+</sup>/CD34<sup>+</sup> cellen. De toename van AAT spiegels is waarschijnlijk het gevolg van hoge spiegels van proteases zoals NE, dat gebonden wordt door AAT. Proteases spelen een belangrijke rol bij HSPC mobilisatie. Echter, in studies met muizen die op experimentele wijze een of meerdere proteases missen, wordt wel HSPC mobilisatie gezien door G-CSF. Deze data en de gegevens uit onze studie tonen aan dat er waarschijnlijk zowel protease-afhankelijke als protease-onafhankelijke mechanismen bestaan die, onder invloed van cytokines, leiden tot mobilisatie van HSPC van beenmerg naar bloed.

## HSPC MOBILISATIE EN DE BEENMERG NICHE

Recent onderzoek heeft aangetoond dat de beenmergniche bestaat uit hematopoietische en niet-hematopoietische cellen, die een belangrijke rol spelen in de mobilisatie van HSPC. Mesenchymale stromale cellen (MSC) zijn niet-hematopoietisch en hebben de capaciteit om te differentiëren naar botcellen, vetcellen en kraakbeencellen. In het beenmerg vormen MSC een onderdeel van de HSC niches die zich rond verschillende typen bloedvaten bevinden. MSC hebben een belangrijke rol in het HSC micromilieau. Daarnaast spelen zij ook een rol bij het nestelen van de HSC in het beenmerg na een stamceltransplantatie. Dat vormde de aanleiding voor de onderzoeken die in **hoofdstuk 5** zijn beschreven, waarbij wij het effect van de toediening van MSC op het beenmerg hebben bestudeerd.

Wij toonden hierbij aan dat dagelijkse toediening van MSC bij muizen leidt tot toegenomen celcyclus activiteit van de HSC. Daarnaast werd na toediening van MSC ook een significant verlaagd aantal macrofagen gezien, zowel bij macrofagen die langs het endosteum liggen als bij CD169<sup>+</sup> macrofagen. Factoren zoals *Cxcl12* en *Vcam*, die zorgen dat HSC in het beenmerg blijven, waren significant verlaagd in endosteale cellen. Al deze veranderingen zijn gelijk aan wat gezien wordt in het beenmerg na mobilisatie met G-CSF. Toediening van MSC leidt echter op zichzelf niet tot mobilisatie, maar bij toediening van MSC en G-CSF samen werden meer dan twee keer zoveel gemobiliseerde HSPC gezien. Daarnaast liet gezamenlijke toediening van MSC en G-CSF, vergeleken met G-CSF alleen, ook een afname zien in *Cxcl12*, *Vcam* en *Scf* in endosteale cellen. Met behulp van bioluminescentie werd aangetoond dat bij muizen waarbij MSC in een ader ingespoten werd, de MSC vastliepen in de longen en niet terugkeerden naar het beenmerg. Wanneer de kweekvloeistof van MSC werd toegediend samen met G-CSF werd vergelijkbare mobilisatie van HSPC gezien. Hieruit leidden wij af dat MSC hun effect op de HSC niche waarschijnlijk uitoefenen door middel van een oplosbare stof. Omdat bekend is dat MSC kleine blaasjes kunnen uitscheiden (extracellulaire vesikels, EV), hebben wij onderzocht of het deze EV waren, die via de kweekvloeistof van de MSC, G-CSF geïnduceerde HSPC mobilisatie versterkten. Het bleek inderdaad dat toediening van uit MSC verkregen EV dezelfde effecten liet zien als wanneer alleen MSC toegediend werden: toegenomen HSPC mobilisatie bij combinatie met G-CSF gedurende 3 dagen, toegenomen celcyclus activiteit van de HSPC en een afname van de expressie van *Cxcl12*, *Vcam* en *Scf*. Deze data laten zien dat MSC, via de uitscheiding van EV, een situatie in het beenmerg creëren waarbij factoren, die de stamcellen in het beenmerg verankeren, verlaagd zijn en de HSPC zich in verhoogde celcyclus activiteit bevinden. Deze situatie leidt bij toediening van G-CSF tot meer dan een verdubbeling van het aantal gemobiliseerde HSPC. Verder onderzoek is nodig om aan te tonen welke factoren die zich in de EV bevinden, verantwoordelijk zijn voor deze waarnemingen.

Niet alleen MSC en macrofagen, maar ook andere bindweefsel - en hematopoietische cellen, zoals rode bloedcellen, vaatcellen en cellen van het sympatische zenuwstelsel vormen de HSC niche in het beenmerg. De complexe interacties tussen HSPC en deze celpopulaties worden beschreven in **hoofdstuk 6**. Een uitgebreid arsenaal aan cytokines, chemokines en kleine moleculen is betrokken bij hematopoiese onder fysiologische omstandigheden en bij HSPC mobilisatie.

Enkele van deze stoffen, zoals G-CSF, spelen een belangrijke rol bij de toepassing van HSPC mobilisatie in de klinische praktijk. De toepassing van deze stoffen wordt beschreven in dit hoofdstuk.

Er zijn grote stappen gezet in het ophelderen van de complexe mechanismen die ten grondslag liggen aan HSPC mobilisatie. Er is echter nog steeds behoefte aan innovatieve middelen en behandelingen die optimale mobilisatie van HSPC mogelijk maken. Verder onderzoek hiernaar zal hopelijk leiden tot betere, veiliger en meer efficiënte mobilisatiestrategieën.





# CHAPTER 8

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## DANKWOORD

Dit proefschrift had zonder een groot aantal personen nooit geschreven kunnen worden. Dit is de plek om hen, naar Leidse mores kort maar krachtig, te danken voor de steun in de afgelopen jaren.

Wim Fibbe: ooit ben jij een onderzoekslijn naar stamcelmobilisatie gestart en ik ben blij dat ik daar een groot aantal jaren deel van heb mogen uitmaken. Ik heb veel van je geleerd, niet alleen van je compromisloze aanpak van de wetenschap, maar ook van je bestuurlijke en diplomatieke talenten. Ons lokt nu beiden *la dolce vita*...

Melissa van Pel: door jouw continue steun, door dik en dun, heeft dit proefschrift eindelijk het licht gezien. Ook al vind je dat dokters eigenlijk niet geschikt zijn voor basaal wetenschappelijk onderzoek, jouw (virtuele) zweepje heeft me tot deze eindstreep gebracht.

Mijn opleiders, Rob Bieger (†), Edo Meinders en Roel Willemze: dank omdat jullie mij tot de dokter gemaakt hebben die ik nu ben.

De analisten wil ik danken voor jullie geweldige hulp bij de talloze experimenten, in het bijzonder Henny Hagoort, Joost van den Muijsenberg en Rob Zuijderduijn.

Dank aan de collega-onderzoekers Gonnie Alkemade, Ronald van Os en Jan Stolk: zonder jullie zouden de artikelen in deze vorm niet bestaan hebben.

Alle studenten die tijdens hun stages hebben geholpen met het uitvoeren van experimenten en het verzamelen van de data ben ik veel dank verschuldigd; in het bijzonder Donnée, Ingmar, Kim, Abdel, Oliver, Christiaan & Christiaan en Marjolein.

Mijn (oud-)collega's in Ede: dank voor de kansen die jullie mij gaven om dit onderzoek naast mijn drukke bezigheden te kunnen blijven doen.

Mijn paranimfen: dank voor de steun op deze spannende dag.

Mijn (nieuwe) collega's in Amsterdam: beloofd is beloofd, hier ligt dan de afronding van vele jaren werk. Ook in het OLVG is er nog genoeg te doen, dus ik zal mij niet vervelen.

Mijn collega's in het NIV-bestuur, Bas en alle medewerkers van het Bureau: dankzij jullie lukt het om alle bordjes in de lucht te houden.

Erwin, enorm veel dank dat je de cover van mijn proefschrift wilde maken. Jouw kunstenaarschap staat op eenzame hoogte.

Dank aan mijn lieve familie en schoonfamilie die mij altijd heeft gesteund: mijn moeder die er nooit aan getwijfeld heeft dat ik dit ooit af zou maken, Frans, Annet, Nienke, Gerdien, Merijn, Emiel & alle aanhang die dat misschien wel deden, maar nooit hebben laten merken.

Lieve Gabriël: samen met jou ontdek ik de wereld, van Amsterdam naar Parijs naar New York naar Città della Pieve en weer terug. Sinds dit jaar ook "officieel". Ik kan alleen maar hopen op vele jaren meer.

## CURRICULUM VITAE

De auteur van dit proefschrift, Evert-Jan de Kruijf, werd op 30 juni 1965 geboren in Gemert (N.B.). Hij groeide op in Reeuwijk en haalde in 1983 zijn VWO diploma aan het St.-Antoniuscollege in Gouda. In dat jaar begon hij ook aan de studie Geneeskunde aan de Rijksuniversiteit Leiden. Zijn propaedeuse haalde hij in 1984, gevolgd door het doctoraalexamen in 1989. Tijdens deze periode deed hij onderzoek bij de afdeling Oogheelkunde van het Academisch Ziekenhuis Leiden (1987, dr. J.A. van Best) en bij de afdeling Anesthesiologie van de University of Utah, Salt Lake City, Utah (1988-1989, prof. D.R. Westenskow, Ph.D.).

In 1992 behaalde hij zijn arts-examen (*cum laude*), waarna hij zijn militaire dienstplicht vervulde als reserve-eerste-luitenant-arts bij de Koninklijke Landmacht. In 1994 startte hij zijn opleiding tot internist in het Bronovo Ziekenhuis (dr. R. Bieger), gevolgd door het academische deel in het Leids Universitair Medisch Centrum van 1997 tot 2000 (prof. dr. A.E. Meinders), waarna hij op 1 januari 2000 werd ingeschreven als internist. Vanaf 2000 werkte hij gedurende 4 jaar als fellow bij de afdeling Hematologie van het LUMC (prof. dr. R. Willemze), waar hij op het Laboratorium voor Experimentele Hematologie onder leiding van prof. dr. W.E. Fibbe startte met het in dit proefschrift beschreven onderzoek.

In 2004 vestigde hij zich als internist-hematoloog in Ziekenhuis Gelderse Vallei in Ede, waar hij tot 2018 naast zijn klinische werkzaamheden verschillende functies vervulde, waaronder voorzitter van de Vereniging Medische Staf van 2012-2016. Daarnaast is hij bestuurlijk actief op het terrein van de interne geneeskunde op nationaal en internationaal niveau, voor onder andere de Nederlandse Internisten Vereniging (sinds 2016 als voorzitter), de European Federation of Internal Medicine en de American College of Physicians (als Honorary Fellow).

Sinds 2018 is hij als internist-hematoloog verbonden aan het OLVG in Amsterdam.

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# LIST OF ABBREVIATIONS

|            |                                                                           |
|------------|---------------------------------------------------------------------------|
| A2M        | alpha-2-macroglobulin                                                     |
| AAT        | alpha-1-antitrypsin                                                       |
| AATD       | alpha-1-antitrypsin deficiency                                            |
| ACT        | alpha-1-antichymotrypsin                                                  |
| Ang-1      | angiopoietin 1                                                            |
| B/NK       | B and NK cell progenitor                                                  |
| BFU-E      | burst-forming units-erythroid                                             |
| BM         | bone marrow                                                               |
| BOP        | N-(benzenesulfonyl)-L-prolyl-L-O-(1-pyrrolidinylcarbonyl)tyrosine         |
| CAFC       | cobblestone area-forming cell                                             |
| CAR cell   | CXCL12-abundant reticular cell                                            |
| CaSR       | calcium-sensing receptor                                                  |
| CFC        | colony-forming cell                                                       |
| CFU-E      | colony-forming units-erythroid                                            |
| CFU-G      | colony-forming units-granulocytes                                         |
| CFU-GEMM   | colony-forming units-granulocytes/erythrocytes/macrophages/megakaryocytes |
| CFU-GM     | colony-forming units-granulocytes/macrophages                             |
| CFU-M      | colony-forming units-monocytes/macrophages                                |
| CFU-Mk     | colony-forming units-megakaryocytes                                       |
| CFU-S      | colony-forming unit spleen                                                |
| CG         | cathepsin G                                                               |
| CLP        | common lymphoid progenitor                                                |
| CMP        | common myeloid progenitor                                                 |
| COPD       | chronic obstructive pulmonary disease                                     |
| COX-2      | cyclooxygenase-2                                                          |
| CRP        | C-reactive protein                                                        |
| CRU        | competitive repopulation unit                                             |
| CSF        | colony-stimulating factors                                                |
| CXCL4      | C-X-C chemokine ligand 4                                                  |
| CXCL12     | C-X-C chemokine ligand 12                                                 |
| CXCR4      | C-X-C chemokine receptor type 4                                           |
| DPP4       | dipeptidyl peptidase-4                                                    |
| EC         | endothelial cells                                                         |
| EGF-TM7    | epidermal growth factor-seven transmembrane                               |
| EPCR       | endothelial protein C receptor                                            |
| EP4        | prostaglandin E2 receptor 4                                               |
| E-selectin | endothelial-leucocyte adhesion molecule 1                                 |
| ETP        | early thymic progenitor                                                   |
| EV         | extracellular vesicles                                                    |
| FACS       | fluorescence-activated cell sorting                                       |

|                  |                                                                                 |
|------------------|---------------------------------------------------------------------------------|
| FBS              | fetal bovine serum                                                              |
| FcγRI            | Fc γ receptor I                                                                 |
| FITC             | fluorescein isothiocyanate                                                      |
| Flt3 ligand / FL | fms-like tyrosine kinase 3 ligand                                               |
| G-CSF            | granulocyte colony-stimulating factor                                           |
| G-CSF-R          | granulocyte colony-stimulating factor receptor                                  |
| GFAP             | glial fibrillary acidic protein                                                 |
| GFP              | green fluorescent protein                                                       |
| GM-CSF           | granulocyte-macrophage colony-stimulating factor                                |
| GMP              | granulocyte macrophage progenitor                                               |
| HIV              | human immunodeficiency virus                                                    |
| HPC              | hematopoietic progenitor cell                                                   |
| HSC              | hematopoietic stem cell                                                         |
| HSC/HPC          | hematopoietic stem and progenitor cell                                          |
| HSPC             | hematopoietic stem and progenitor cell                                          |
| ICAM-1           | intercellular adhesion molecule-1                                               |
| IFN-γ            | interferon-gamma                                                                |
| IL-1             | interleukin-1                                                                   |
| IL-8             | interleukin-8                                                                   |
| i.p.             | intraperitoneally                                                               |
| IT-HSC           | intermediate-term hematopoietic stem cell                                       |
| KLS              | c-Kit <sup>hi</sup> Lin <sup>neg</sup> Sca-1 <sup>pos</sup>                     |
| KTLS             | c-Kit <sup>hi</sup> Thy-1 <sup>lo</sup> Lin <sup>neg</sup> Sca-1 <sup>pos</sup> |
| LECT2            | leukocyte cell-derived chemotaxin 2                                             |
| LEPR             | leptin receptor                                                                 |
| LFA-1            | leukocyte function-associated antigen-1                                         |
| Lin-             | lineage negative                                                                |
| LMPP             | lymphoid-primed progenitor                                                      |
| LSK              | c-Kit <sup>hi</sup> Lin <sup>neg</sup> Sca-1 <sup>pos</sup>                     |
| LTC-IC           | long-term culture-initiating cell                                               |
| LT-HSC           | long-term hematopoietic stem cell                                               |
| MACS             | magnetic-activated cell sorting                                                 |
| MAFIA            | macrophage-Fas-induced apoptosis                                                |
| MASP             | MBL-associated serine protease                                                  |
| MBL              | mannan-binding lectin                                                           |
| M-CSF            | macrophage colony-stimulating factor                                            |
| MEP              | megakaryocyte erythrocyte progenitor                                            |
| MGK              | megakaryocyte                                                                   |
| MIP-2            | macrophage inflammatory protein-2                                               |
| MLP              | multilymphoid progenitor                                                        |
| MMP-9            | matrix metalloproteinase-9                                                      |

|                |                                                         |
|----------------|---------------------------------------------------------|
| MNC            | mononuclear cell                                        |
| MPP            | multipotent progenitor cell                             |
| MSC            | mesenchymal stromal cell                                |
| NE             | neutrophil elastase                                     |
| Nes            | Nestin                                                  |
| NG2            | neuron/glial antigen 2                                  |
| NK             | natural killer                                          |
| NOD/SCID       | nonobese diabetic/severe combined immunodeficient       |
| NPY            | neuropeptide Y                                          |
| OB             | osteoblast                                              |
| OPG            | osteoprotegerin                                         |
| osteomacs      | osteal tissue macrophages                               |
| PB             | peripheral blood                                        |
| PBMC           | peripheral blood mononuclear cells                      |
| PBS/BSA        | phosphate-buffered saline/bovine serum albumin          |
| PDGFRA         | platelet-derived growth factor receptor alpha           |
| PE             | phycoerythrin                                           |
| PGE2           | prostaglandin E2                                        |
| PLC- $\beta$ 2 | phospholipase C- $\beta$ 2                              |
| proB           | pro-B-cell                                              |
| qRT-PCR        | quantitative real time-polymerase chain reaction        |
| RANKL          | receptor activator of nuclear factor kappa-B ligand     |
| rhG-CSF        | recombinant-human granulocyte colony-stimulating factor |
| Rho            | Rhodamine-123                                           |
| rmG-CSF        | recombinant-mouse granulocyte colony-stimulating factor |
| ROS            | reactive oxygen species                                 |
| S1P            | sphingosine-1-phosphate                                 |
| Sca-1          | stem cell antigen-1                                     |
| SCF            | stem cell factor                                        |
| SD             | standard deviation                                      |
| SDF-1          | stromal cell-derived factor 1                           |
| SEM            | standard error of the mean                              |
| SLAM           | signaling lymphocyte activation molecule                |
| SLPI           | secretory leukocyte proteinase inhibitor                |
| SNS            | sympathetic nervous system                              |
| SP             | side population                                         |
| ST-HSC         | short-term hematopoietic stem cell                      |
| TBI            | total body irradiation                                  |
| TGF- $\beta$ 1 | transforming growth factor beta-1                       |
| TIMP           | tissue inhibitors of metalloproteinases                 |
| TNF- $\alpha$  | tumor necrosis factor alpha                             |
| TPO            | thrombopoietin                                          |
| VCAM-1         | vascular cell adhesion molecule-1                       |

|       |                     |
|-------|---------------------|
| VLA-4 | very late antigen-4 |
| VLA-5 | very late antigen-5 |
| WBC   | white blood cells   |

*"Blood is that fragile scarlet tree we carry within us"*

— Sir Osbert Sitwell, *The Scarlet Tree*, 1946. —



