Biology of cytokine-induced hematopoietic stem and progenitor cell mobilization
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CHAPTER 1

INTRODUCTION

“Blut ist ein ganz besondrer Saft”
— Johann Wolfgang von Goethe, Faust I, verse 1740. —

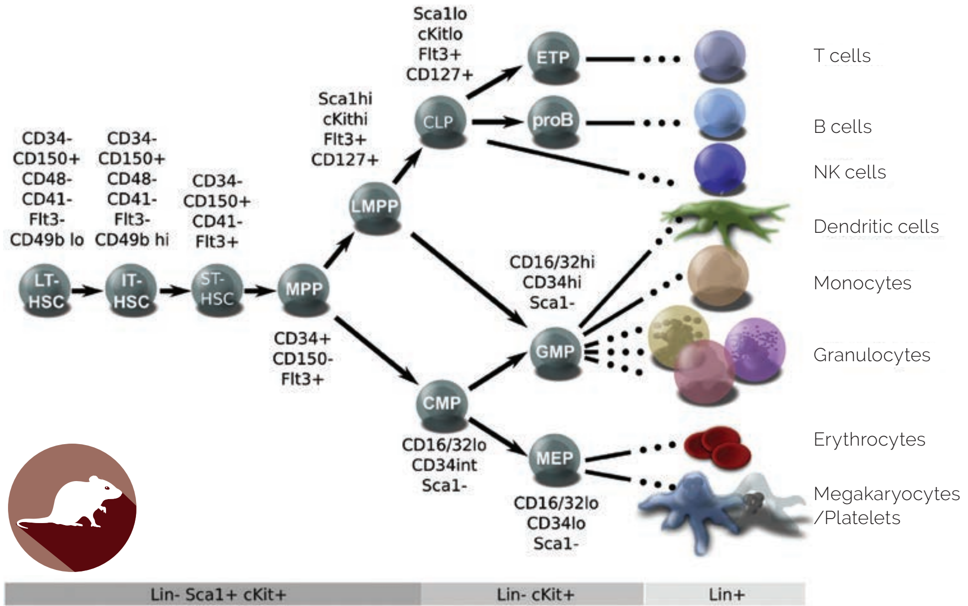
HEMATOPOIESIS

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.[1] 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.[2] 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.[2] 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.[3] 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.[4,5] 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.[6]

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).[7] A subgroup of HSC, named intermediate-term HSC (IT-HSC), is capable of engraftment beyond 12 weeks but becomes extinct between 6-8 months.[8] 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).[7,9] 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.[7] 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



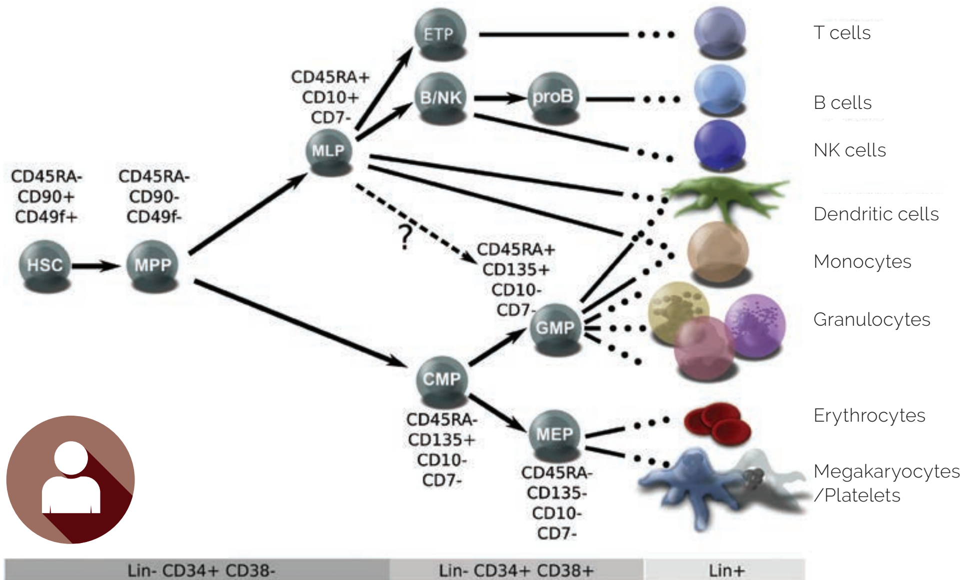


FIGURE 1. **Hierarchical model of lineage determination in murine and human hematopoiesis.**

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.[10] 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.[11]

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).[12] This population of $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ (LSK) cells comprises around 0.1% of the total bone marrow cells.[13] 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.[14] Cells in this "KTLS" population were shown to be the only cells in murine bone marrow that can gave rise to long-term multilineage repopulation in syngeneic and allogeneic transplantation.[15]

The CD2/signaling lymphocyte activation molecule (SLAM) family of receptors has been shown to further phenotypically differentiate HSPC.[16] The cell surface receptors CD150 and CD244 and its ligand CD48 can be used to reliably differentiate between highly purified HSC ($CD150^+/CD244^-/CD48^-$), multipotent progenitors ($CD150^-/CD244^+/CD48^-$) and oligopotent progenitors ($CD150^-/CD244^+/CD48^+$).[16,17] 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).[12]

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.[18]

Micro-array analysis of the genes expressed by murine HSC demonstrated increased expression of the gene encoding the murine endothelial protein C receptor (EPCR).[19] 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^{+}/CD150^{+}/CD48^{-}/CD45^{-}), significantly enriches murine HSC.[19,20]

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.[21] 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.[21] In combination with the SLAM marker CD150, SP cells can be further divided into functionally different populations of long-term repopulating cells.[22] Rhodamine-123 (Rho) is a fluorescent dye that accumulates intracellularly and binds to mitochondria with increasing intensity as cells become activated.[23,24] Cells with less active mitochondria exhibit a reduced uptake of Rho. Rho staining of HSPC allows for the separation of Rho^{+}(dull) cells with long-term repopulating capacity and Rho^{++}(bright) cells, representing HSC and HPC, respectively.[23,24]

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^{+}.[25–27] However, human bone marrow CD34^{+} cells are heterogeneous, since this population not only contains HSC but also differentiated progenitors.[28] For clinical purposes, CD34 is widely used as a cell surface marker to identify and isolate human HSPC because the number of CD34^{+} cells in a hematopoietic cell transplant correlates with the rate of hematopoietic reconstitution.[29]

The purification of human HSC requires the exclusion of mature lineage markers (Lin^{-}) such as CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b and GlyA.[30] The use of additional surface markers, such as CD38, CD45RA, Thy1 and integrin α6 (ITGA6, CD49f), can further enrich a cell population for HSC.[30,31] This improvement is illustrated by the observation that in an experimental setting, a single Thy1^{+}Rho^{lo}CD49f^{+} human bone marrow cell is capable of long term multilineage engraftment.[31]

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.[32]

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_2 conditions.[33,34] 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).[35,36] 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).[36] 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.[17]

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.[37-39] 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.[37,40] Ploemacher et al. have developed a quantitative CAFC assay by using limited dilution and Poisson statistics to assess the frequency of HSPC.[37,40] In this assay, day 7 CAFC correlate with HPC, whereas day 28 CAFC correlate with HSC that show long-term *in vivo* repopulating capacity.[37,40] The disadvantages of both systems are that they are time-consuming and difficult to standardize.[41] Originally developed to study murine hematopoiesis, both assays have been adapted to also examine human hematopoiesis.[42,43]

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.[17,44]

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.[36] The tested cell population is transplanted into irradiated recipients along with 1-2 x 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.[17] These cells provide short-term radioprotection and make the quantification of the HSC frequency possible if limiting dilutions of test cells are transplanted.[45] 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 x 10^5 recipient cells) or too many (>2 x 10^7 recipient cells) HSC are present, the data may be less reliable.[36,46] Another limitation is its dependence upon homing and engraftment processes, which may be disturbed without altering stem cell function per se.[36] 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.[47,48]

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.[17]

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.[17]

Human HSC are studied by transplantation of human hematopoietic cells into immune-deficient mice.[49] 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 $CD34^+$/$CD38^-$ cells, called SCID-repopulating cells (SRC).[50] 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-γ-chain (NSG-mouse) results in the complete loss of B, T

and NK cells.[51] NSG mice engrafted with human HSC show significantly increased numbers of human CD45$^+$ cells in the host bone marrow in comparison with similarly treated NOD/SCID mice.[51,52] 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.[53]

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.[54] 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.[54] The adult bone marrow is a highly vascularized tissue, with arterioles transitioning to venous sinusoids in close proximity to the endosteum.[55] During homeostasis, the vast majority of HSC reside in the bone marrow, with only a small minority of HSC present in the circulation.[56] 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.[57] 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.[58,59] 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.[58]

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).[60-64] 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.[60,65,66]

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.[67,68] 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.[69,70] 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.[71-74] Also, recent *in vivo*

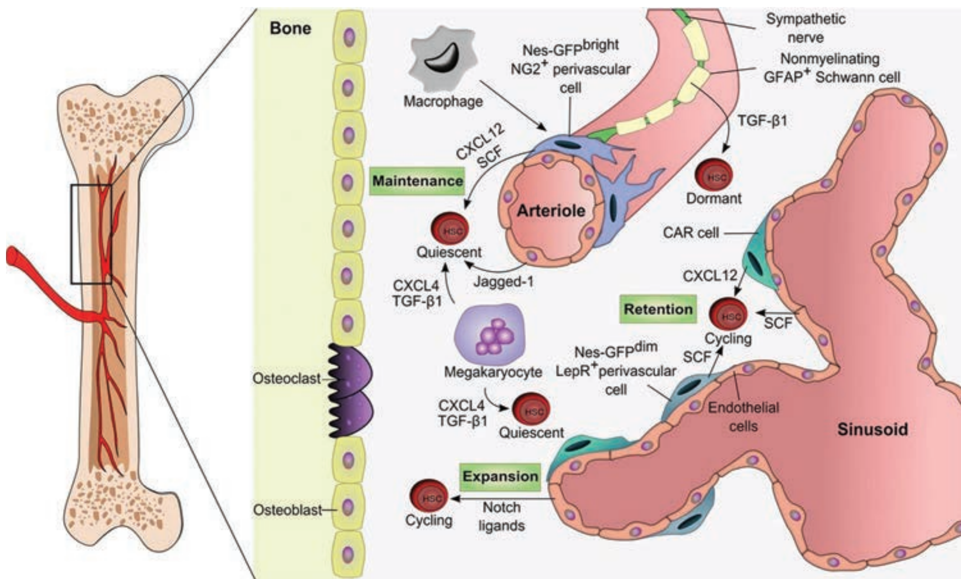


FIGURE 2. The adult bone marrow HSC niche.

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.[69,71,75–77] 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.[78,79] 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.[80,81] 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).[82]

Additionally, these perivascular MSC differentially express one or more of the following markers: neuron/glial antigen 2 (NG2), leptin receptor (LEPR) and nestin, an intermediate filament protein known as a marker for neural stem cells.[83–85] NG2^{+}/LEPR^{-}/nes^{bright} MSC are generally located around arterioles, close to the endosteum, and NG2^{-}/LEPR^{+}/nes^{dim} reticular cells are mostly situated around the sinusoids, away from the endosteal surface.[83] Hematopoietic stem cells commonly co-localize with nestin-GFP transgene (Nes-GFP)$^{+}$ cells, suggesting that HSC prefer to localize close to arterioles.[83,84]
Perivascular cells, which express CXCL12, a chemokine responsible for the retention of HSC in the bone marrow, co-localize with HSC adjacent to sinusoids.[76] These CXCL12-abundant reticular (CAR) cells might overlap in function with LEPR^{+} perivascular cells, which also express CXCL12.[76,78,85] Deep confocal imaging studies indicate that nearly all HSC co-locate with LEPR^{+} and CXCL12^{high} cells, indicating the essential role of these cells in the niche.[77] LEPR^{+} 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.[86] 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.[86] 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.[78,79] 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.[87-89]
In the perivascular niche, Nes-GFP^{+} cells are closely associated with sympathetic nerve fibers and express the β3-adrenergic receptor.[84] 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.[84,90] Sympathetic nerve fibers are sheathed by non-myelinating Schwann cells that are Nes^{+}, 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.[91] Damage to the SNS from neurotoxic chemotherapy results in impaired hematopoietic regeneration, due to the selective loss of adrenergic innervation.[92] Interesting from a clinical standpoint is the finding that neuroprotection in mice treated with chemotherapy can rescue bone marrow engraftment and mobilization.[92,93]
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.[94–96] Several macrophage populations in the bone marrow have been described in terms of their surface antigen expression, location and function.[96] 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.[96,97]

Osteal tissue macrophages (osteomacs) are F4/80$^+$ cells that regulate osteoblast function by forming a canopy over bone-lining osteoblasts.[98] 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.[94]

CD169$^+$ macrophages were identified as critical stromal niche supportive cells that indirectly regulate HSC cycling and HSC pool size.[95,99] Using macrophage-specific conditional depletion models, reductions in bone marrow CD169$^+$ 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$^+$ niche cells and the migration of HSPC to the bloodstream.[95] Under steady state conditions, the depletion of bone marrow resident macrophages increases both HSC proliferation and the absolute number of quiescent HSC.[99] Furthermore, CD169$^+$ macrophages are essential for supporting erythropoiesis by forming erythroblastic islands, where a central macrophage is surrounded by erythroid precursors in varying stages of development.[100] 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.[101,102] In addition, CD169$^+$ macrophages are essential to the circadian fluctuation in circulating HSC, as they are required for the clearance of aged (CD62^{lo}/CXCR4^{hi}) neutrophils, which in turn results in decreased CXCL12 expression and subsequent HSC mobilization.[103]

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.[61,63] Interestingly, around 20% of phenotypic HSC co-localize with MGK.[63] Megakaryocytes play a role in HSC

maintenance by producing CXCL4 (or platelet factor 4), transforming growth factor beta-1 (TGF-β1) and TPO.[104-106] The depletion of MGK results in the activation of quiescent HSC and increased HSC proliferation through reduced levels of biologically active TGF-β1 in the bone marrow, suggesting a supportive role of MGK in the HSC niche (Figure 2).[104,105]

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.[107] It was subsequently found that CFU-GM were also increased in the recovery phase after chemotherapy.[108] 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).[109] 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.[110] 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).[111,112] 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.[112] In 1983, the purification of murine G-CSF was established, which was followed the next year by the purification of human G-CSF.[113,114]

Granulocyte colony-stimulating factor primarily affects hematopoietic cells of the myeloid lineage by stimulating the proliferation and differentiation of these cells.[115] After binding to its receptor, G-CSF activates the phosphorylation of the Ras/MAP kinase pathway and the protein tyrosine kinases JAK1 and JAK2.[116,117] In granulocytes, G-CSF enhances chemotaxis and superoxide production, leading to an increased response to antigens.[118] The G-CSF protein is encoded with a single gene, in humans located on chromosome 17q11-22.[119] G-CSF knockout mice have reduced numbers of granulocytes, macrophages and their precursors, leading to an inability to respond to bacterial infections.[120] 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.[121]
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.[113,114] 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 deducted, 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.[122,123] 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.[124] 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 x 10^9/l, with the neutrophils functioning normally *in vitro*.[125] In autologous bone marrow transplantation, G-CSF was shown to shorten the period of neutropenia after myeloablative chemotherapy or total body irradiation.[126]
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.[127] 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.[128,129] Patients with congenital neutropenia also benefited from the administration of G-CSF.[130] In over 90% of the patients with severe chronic neutropenia, the administration of G-CSF induced an increase of neutrophils >1.0 x 10^9/l, resulting in a reduction of bacterial infections and a diminished use of antibiotics.[131]
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.[132] A similar effect on peripheral

blood HSPC was seen after the administration of GM-CSF in cancer patients.[133] 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.[134–137] In 1992, Sheridan et al. showed that patients receiving G-CSF-mobilized peripheral blood progenitors after high-dose chemotherapy had significantly faster hematopoietic reconstitution.[138] 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.[109]

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.[139] 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.[139] 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.[140] When administered locally, IL-8 leads to an inflammatory response characterized by neutrophil accumulation and plasma protein extravasation.[141] Interleukin-8 can be induced by the chemokine interleukin-1 (IL-1), which was shown to rapidly mobilize HSPC in mice.[142] 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.[143–145] 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.[144] 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.[144]

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.[146] FL binds to the tyrosine kinase type III receptor Flt3/Flk2 (CD135), which is expressed by HPC.[147] In steady state hematopoiesis, FL is important for lymphocyte development, but not for differentiation into myeloid lineages.[148] 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.[146,149-153]

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.[146,153] 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.[146] 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.[153-155]

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.[156] 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.[156] Neutrophils also play an essential role in HSPC mobilization with the chemokines GROβ/CXCL2 and GROβT/CXCL24.[157] Mice that are deficient for the G-CSF-R (G-CSF-R$^{-/-}$) are neutropenic and do not mobilize after stimulation with IL-8 or cyclophosphamide, suggesting that G-CSF-R^{+} neutrophils are required.[158] In mice that are chimeric for wild-type and G-CSF-R$^{-/-}$ bone marrow cells, treatment with G-CSF leads to the mobilization of equal proportions of both G-CSF-R$^{-/-}$ and G-CSF-R$^{+/+}$ HSPC.[159] 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⁺ cells of the monocytic lineage.[160] 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.[160]

THE ROLE OF INTEGRINS IN HSPC ENGRAFTMENT AND MOBILIZATION

Integrins are heterodimeric proteins that consist of a non-covalently bound α - and β-subunit that mediate cell-cell and cell-extracellular matrix interactions. Thus far, 18 α-chains and 8 β-chains have been identified, and each combination of α- and β-subunits leads to adhesion molecules with differential ligand specificity.[161] The integrin subunits α4, α5, α6, α9 and β1 are expressed on the surface of HSPC, while α1-α3, α8, α10 and α11 have not been detected on HSPC.[162–164] Beta-1-integrins are involved in HSC-MSC adhesion, as indicated by co-cultures of human bone marrow-derived MSC and CD34⁺ HSPC.[162,165] *In vivo*, HSC transplantation experiments have shown that various integrins, such as LFA-1 (leukocyte function-associated antigen-1, αLβ2 integrin, CD11a/CD18), VLA-4 (α4β1-integrin) and VLA-5 (α5β1-integrin) are involved in the engraftment of HSC in mice and humans.[166,167] Besides their role in HSC engraftment, integrins are also involved in HSPC retention and mobilization from the bone marrow.[168–171] Our group showed that after a single injection of neutralizing anti-LFA-1 antibodies, IL-8 induced mobilization of HSPC in mice is blocked.[172] Moreover, injection of neutralizing antibodies to the LFA-1 ligand intercellular adhesion molecule-1 (ICAM-1) significantly inhibited IL-8 induced HSPC mobilization.[172] 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.[173] The administration of antibodies against the α-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.[174] 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.[174]

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.[170,171] 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.[175–178] Natalizumab is a recombinant humanized monoclonal antibody against the α4 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⁺ cells

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

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.[183,184] 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.[185] Subsequently, VCAM-1 is cleaved by NE and CG, causing an increase in soluble VCAM-1 in the peripheral blood.[186] 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.[187,188]
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.[189]
Proteolytic activity also affects the interaction between SCF and its receptor c-Kit (CD117).[190] 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.[190] Cleavage of SCF by MMP-9 has been reported during treatment with the chemotherapeutic agent 5-fluorouracil.[191]
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 $CD34^+$ mobilization.[192]
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.[193] 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.[194] Therefore, the physiologic significance of proteases in stem cell mobilization remains uncertain.[195].

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.[196,197]
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.[196] Both AAT and ACT are observed in bone marrow extracellular fluids.[198] AAT is locally produced, mainly by osteoblasts, suggesting a protective role for this protease inhibitor in the HSC niche.[199] Matrix metalloproteinases are inhibited by so-called tissue inhibitors of metalloproteinases (TIMPs).[200] Although MMPs play a role in HSPC mobilization, the role of TIMPs in HSPC mobilization is probably less critical.[157,189,201]

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.[198] 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.[202] 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.[202] 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.[201–203]

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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