REVIEW

Mobilized peripheral blood: an updated perspective [version 1; peer review: 2 approved]

Darja Karpova1, Michael P. Rettig2, John F. DiPersio2

1Division of Stem Cells and Cancer, German Cancer Research Center (DKFZ) and DKFZ-ZMBH Alliance, Heidelberg, 69120, Germany
2Division of Oncology, Department of Medicine, Washington University School of Medicine, St. Louis, Missouri, 63110, USA

Abstract

Enforced egress of hematopoietic stem cells (HSCs) out of the bone marrow (BM) into the peripheral circulation, termed mobilization, has come a long way since its discovery over four decades ago. Mobilization research continues to be driven by the need to optimize the regimen currently available in the clinic with regard to pharmacokinetic and pharmacodynamic profile, costs, and donor convenience. In this review, we describe the most recent findings in the field and how we anticipate them to affect the development of mobilization strategies in the future. Furthermore, the significance of mobilization beyond HSC collection, i.e. for chemosensitization, conditioning, and gene therapy as well as a means to study the interactions between HSCs and their BM microenvironment, is reviewed. Open questions, controversies, and the potential impact of recent technical progress on mobilization research are also highlighted.

Keywords

Mobilization, Hematopoietic stem and progenitor cells, G-CSF, CXCR4, VLA4, CXCR2, BM niche, Chemosensitization, Conditioning, Gene therapy
Introduction
Discovered by pure chance in patients recovering from chemotherapy almost 45 years ago, the phenomenon of hematopoietic stem cell (HSC) mobilization has transformed the clinical practice of HSC transplantation. It has further extended to indications beyond HSC collection, including mobilization-based chemosensitization, conditioning, and gene therapeutic approaches, which are areas of intensive research. Better understanding of the pathways governing HSC trafficking can provide important insights into how stem cell localization within the bone marrow (BM) is regulated, which explains a continued need for basic research on mobilization to define the underlying molecular and cellular mechanisms.

In mammals, the first definitive HSCs arise in several intra and extraembryonic tissues from which they first migrate into the fetal liver. Following expansion in the fetal liver, HSCs continue their journey towards the BM, where the overwhelming majority of adult HSCs are subsequently found in their unique, specialized environments, the BM niches. Interestingly, despite the dramatically reduced migratory activity upon BM colonization, a small fraction of adult HSCs can be found in the peripheral circulation at any given time. Even though random leakiness of BM retention pathways cannot be excluded as a cause, the regularity of this physiological HSC egress implies a biological function. The number of HSCs in the circulation at steady state can be substantially augmented by a wide variety of endogenous and exogenous stimuli such as growth factors, chemotherapy, mobilizing agents discovered and tested in preclinical models, only G-CSF alone (healthy donors) or in conjunction with chemotherapy and plerixafor (patients) is being used in the clinic.

Clinically Used:
- G-CSF with or w/o chemotherapy
- G-CSF with or w/o plerixafor

Figure 1. Mobilization stimuli. A wide variety of stimuli that lead to increased numbers of circulating hematopoietic stem cells (HSCs) have been identified, including but not limited to growth factors (cytokines), granulocyte colony-stimulating factor [G-CSF], granulocyte-macrophage colony-stimulating factor [GM-CSF], stem cell factor [SCF], FLT3 ligand [FLT3L], thrombopoietin [TPO], angiopoietin [Angpt], vascular endothelial growth factor [VEGF], interleukins [ILs], chemokines (CXCL12 and analogs), chemokine receptor antagonists (CXCR4 antagonists), bioactive lipids (sphingosine-1 phosphate [S1P] and ceramide-1 phosphate [C1P]), and bacterial toxins (lipopolysaccharide [LPS], and pertussis toxin [PTX]), proteases (trypsin, matrix metalloprotease 9 [MMP9], cathepsin G, and neutrophil elastase) and adenosine receptor agonists (defibrotide) and inhibitors of the intracellular mediator of CXCR4 signaling, the small Rho GTPase Rac1. For the majority of the listed stimuli, a direct or indirect targeting of CXCR4 (green) or VLA4 (blue) signaling or both (red) has been documented. On the systemic level, the time of day, stress, infection, inflammation, exercise, trauma, tissue damage, coagulation system, complement cascade, cortisol, CNS, SNS.

Preclinical Models:
- Cytokines
  - G-CSF, GM-CSF, SCF, FLT3L, TPO, Angpt, VEGF, IL-1, -3, -4, -7, -12, -17, -33
- Chemotaxins
  - CXCL12, Gro-α, -β, γ, IL-8
- Chemokine receptor antagonists
  - CXCR4/CXCL12 antagonists
- Prototoxins
  - Trypsin, CD26, MMP9, Cathepsin G, Neutrophil elastase
- Polysaccharides
  - Dextran, Fucoidan, GAG Mimetetics, Betafectin PGG-Glucan
- Bioactive Lipids
  - S1P, C1P
- VLA4 and VLA4/VLA9 inhibitors
  - VCAM1 and CD44 inhibitors
  - Defibrotide
- Adhesion molecule antagonists
  - Adenosine receptor agonists
  - Ephrin A3 receptor antagonists
- Bacterial Toxins
  - LPS, PTX
- Prostaglandin inhibitors

Time of day, Stress, Infection, Inflammation, Exercise, Trauma, Tissue damage, Coagulation system, Complement cascade, Cortisol, CNS, SNS
mobilization. While the function of homeostatically circulating HSCs remains enigmatic, pharmacologically induced HSC egress is increasingly used as the preferred strategy to generate grafts for HSC transplantation (HSCT), the only curative therapeutic option for many hematopoietic malignancies as well as non-malignant pathologies. HSCT requires the intravenous infusion of a minimum of $2 \times 10^6$ CD34+ stem cells/kg recipient body weight; however, a dose of $5 \times 10^6$ CD34+ cells/kg is considered preferable for early, consistent, and long-term multilineage engraftment. Each failure or delay to collect sufficient hematopoietic stem/progenitor cells (HSPCs) to proceed to transplantation extends the time of high-dose chemotherapy and increases the risk of disease progression in cancer patients.

The need to optimize mobilization regimens with regard to their stem cell yield, side effects and risk profile, cost-effectiveness, and availability for different groups of patients, as well as the need to better understand the communication between HSCs and their niche, continues to drive mobilization research. In this review, we discuss how deciphering the events induced by the most commonly used mobilizing agent, granulocyte colony-stimulating factor (G-CSF), led to the development of new mobilization strategies. We highlight the most recent findings and how we envision the newly discovered mobilization approaches will impact mobilization in the clinic. Alternative applications for mobilization are also reviewed. Lastly, we identify open questions and controversies, prospective directions, and how recent technical advances can be implemented within mobilization research.

Current mobilization regimens

G-CSF-mobilized blood is the preferred graft source for virtually all autologous and an increasing majority of allogeneic HSCTs owing to its generally higher stem cell content, reduced rates of graft failure, and better overall survival as compared to the BM.

After 4–5 days of treatment with G-CSF, circulating HSPCs increase an average of 50–100-fold.

The key role of the stromal compartment in G-CSF-induced mobilization has long been appreciated. Following activation of its G-CSF receptor, BM monocytes/macrophages, the most prominent hematopoietic component of the BM stroma, downregulate several retention molecules, including the major CXCR4 ligand, CXCL12, and several VLA4 ligands by non-hematopoietic stroma, resulting in HSPC egress within minutes.

The long-standing view has been that both HSPC expansion and mobilization are necessary for clinically relevant mobilization. In line with this view, mobilization with CXCR4 or VLA4 antagonists alone fails to achieve numbers that would allow their use without G-CSF, despite promising potential in preclinical models. Very recent findings by our group and others challenge this notion and suggest that efficient recruitment of long-term, serially repopulating HSCs can be accomplished within minutes. Indeed, CXCR4 or VLA4 blockade, when combined with the stimulation of a different chemokine receptor, CXCR2, results in extremely rapid and potent HSC mobilization in mice with a repopulating capacity similar or even superior to G-CSF-recruited HSCs. These observations show that major changes in cellular composition or localization are not required for efficient mobilization. They further highlight the existence of different HSC species that, upon disruption of certain adhesive tethers, can egress from the BM very rapidly with kinetics that appear incompatible with a prior requirement for changes in gene expression.

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Development of alternative HSPC mobilization regimens and grafts

As we continue to learn about the events and mechanisms regulating HSPC egress, we approach the ultimate goal of developing an “optimal” mobilization strategy to collect sufficient numbers of primitive stem cells with superior properties within a day or two. In contrast to the days when clinical observations determined the applicability of a mobilization approach, educated and targeted designs are becoming the basis for the clinical development of mobilizing agents. In addition to the quantity and fitness of the HSPCs, as reflected in their engraftment capacity, the immunogenic properties of the graft (i.e. the graft-versus-host disease (GvHD) profile) are an important feature requiring optimization, potentially at the cost of stem cell numbers. For example, a substantially reduced incidence of GvHD is observed upon transplantation of CXCR4 antagonist-mobilized grafts, possibly due to co-mobilization of a specific population of dendritic cells (DCs) with immunomodulatory properties, plasmacytoid DCs. Along the same lines, grafts mobilized with pegylated G-CSF were superior to standard G-CSF in that they were associated with less GvHD, while graft-versus-leukemia (GvL) effects were improved through mobilization of invariant natural killer T (iNKT) cells. Susceptibility of the mobilized HSPCs to further molecular manipulation, e.g. using gene therapy, is another important criterion when defining the “optimal” mobilization strategy. Lastly, if proven to be suitable for mobilized HSPCs, recently described methods for ex vivo expansion of HSCs are expected to shift the emphasis on HSPC quality over quantity even further.

Studies with CXCR4 and VLA4 antagonists, tested in VLA4 and CXCR4 knockout mice, respectively, implied an independence between the two axes. This suggests that subsets of HSPCs are being retained in the BM by either CXCR4 or VLA4. Combined with the knowledge of the complexity and multiplicity of events induced in the course of G-CSF mobilization, co-existence of these (and possibly other) functionally distinct HSPC populations suggests combinatorial mobilization approaches as the best alternatives to G-CSF. Thus, the small molecule Me6TREN reportedly inhibits CXCR4 and VLA4 signaling simultaneously, possibly through upregulation of the protease MMP9. However, given the controversy regarding the role of MMP9 for mobilization, other approaches should be explored. In addition to cell-intrinsic HSPC retention pathways, disruption of endothelial layer integrity, along with the endothelial cell activation and subsequent crosstalk between endothelial and mature hematopoietic cells, should be included in designing “optimal” mobilization. Recent data suggest that Viagra (sildenafil citrate), a phosphodiesterase type 5 (PDE5) inhibitor which blocks the degradation of cyclic GMP in the smooth muscle cells lining blood vessels, resulting in vasodilation, can synergize with plerixafor to rapidly mobilize stem cells in mice.

Various techniques for ex vivo graft manipulation (e.g. T cell depletion and CD34 enrichment) have been developed that entail extended periods during which the HSPCs stay outside of their natural environment and therefore, unsurprisingly, exhibit reduced stem cell capacity. From further in-depth analyses of differentially mobilized blood (see below), we expect to learn not only how to target specific HSPC populations but also how to mobilize HSPCs without a concurrent mobilization of mature cells, T-cells in particular. In general, a priori cell type-specific targeting remains challenging because of the high conservation of migratory and retention pathways between different hematopoietic cell types. Nevertheless, selective HSPC mobilization represents an intriguing goal that would help reduce additional graft manipulation.
Mobilization beyond stem cell collection

Chemosensitization

In addition to supplying HSPCs with the factors required for their normal development, the BM microenvironment is also a refuge for malignant cells, allowing them to escape cytotoxic therapies and cause disease relapse.465,466 This provides a rationale for targeting the interactions between tumor cells and the BM, with the goal of sensitizing them to therapy. Pathways responsible for the anchorage and survival of malignant cells and resistance to chemotherapy largely overlap with those of normal HSPCs.468,469 Accordingly, blockade of CXCR4 and VLA4 signaling and/or G-CSF was tested in conjunction with chemotherapy in pre-clinical models of acute myeloid leukemia (AML), acute474,475 and chronic476 lymphoid leukemia, and MM.477 Moreover, the FDA-approved CXCR4 antagonist plerixafor has been tested as a chemosensitizing agent alone and in combination with G-CSF in patients with relapsed AML.478,479 While the mobilizing capacity varied substantially, an overall benefit from adding mobilizing agent(s) to chemotherapy has been reported, prolonging survival and decreasing tumor burden490,472,477,480 or even eradicating disease491. The benefits of this approach in AML and other hematologic malignancies, in spite of these preclinical as well as early clinical studies, remain both unclear and controversial.

Conditioning

As HSPCs are pharmacologically driven from the BM into circulation, the temporarily unoccupied spaces (niches) in theory become available to new cells, e.g. the HSPCs introduced into a mobilized recipient during transplantation. The utility of mobilization for non-cytotoxic and on-target conditioning prior to HSCT is supported by the fact that mobilized cells return to the BM after spending some time in peripheral circulation, as shown in studies of parabiotic mice.492 Yet virtually all attempts at mobilization alone for conditioning of an adult host before HSCT have been unsuccessful (Karpova and Rettig, unpublished data). It is unclear whether the reason is that the cells introduced exogenously are inherently disadvantaged (less fit?) compared with endogenously circulating HSPCs or whether the mobilizing agent interferes with the repopulating capacity of the transplanted cells. An intriguing alternative explanation is that owing to targeting/recruitment of a specific population during the mobilization process, and by extension because of emptying of very specific niches, only HSPCs mobilized with the same mobilizing regimen are able to engraft BM niches that become available. Interestingly, since BM- or fetal liver-derived HSPCs have been used to engraft mobilized recipients (Karpova and Rettig, unpublished data), the possibility that a qualitative rather than quantitative approach might lead to successful, persistent engraftment is untested. Given recent reports of successful conditioning using antibody–drug conjugates targeting the pan leukocyte marker CD45 and the CD117-targeting antibodies493–496, or a cocktail of monoclonal antibodies depleting CD47-expressing cells along with T cells, NK cells, and HSPCs497, mobilization-based conditioning may not be a promising approach in postnatal recipients. However, fetal HSPC mobilization with a VLA4 antagonist followed by in utero HSCT results in increased donor HSC homing to the fetal liver and enhanced long-term allogeneic engraftment in mice.498

Therefore, mobilization-based conditioning regimens might be applicable for in utero HSCT.

Gene therapy

Manipulating HSCs to correct mutations that cause inherited diseases of the hematopoietic system such as sickle cell anemia and beta-thalassemia represents a potential cure, with recent advances in gene therapeutic approaches (CRISPR-Cas9, TALEN, and ZFN)499–501 allowing sustainable correction of the genetic defects. Autologous HSCT is the method of choice in this setting, whereby instead of extracting HSCs for subsequent ex vivo manipulation, stem cells are mobilized into the circulation and subjected to gene therapy in situ502–504. As discussed above, HSC collection and ex vivo editing inevitably leads to a diminished stem cell capacity, which can be avoided by editing the cells in the peripheral blood. Proof of principle for mobilization-based gene therapy was reported following mobilization with G-CSF plus a CXCR4 antagonist, with sustained expression of the introduced transgene over a period of 5 months.505 We believe this approach should be developed further, e.g. by using it in combination with mobilization strategies to preferentially recruit stem cells with superior repopulating capacity into the circulation. In addition, these cells may be more susceptible to therapeutic gene editing. Apart from its obvious therapeutic benefit, this approach might become useful for studying functional differences between HSCs that have been mobilized into the circulation and returned to the BM and HSCs that remain in the BM niche.

Biology of the hematopoietic niche

The discovery of compounds and pathways that enable HSPC displacement from the niche has provided important insights into the regulation of HSPC trafficking and maintenance. For example, detailed analysis of the mechanisms underlying G-CSF-induced mobilization was indispensable for establishing monocytes/macrophages as crucial components of the BM niche and understanding their crosstalk with the non-hematopoietic stroma.506,507,508 More recent studies demonstrated that bone marrow dendritic cells regulate endothelial cell function in part through CXCR2 signaling, resulting in HSPC mobilization and loss of bone marrow macrophages.509 Similarly, studies with the CXCR2 ligand tGro-β disclosed the role of another mature leukocyte population, neutrophils, in HSPC trafficking510,511,512. Together with the observation that the circadian release of HSPCs into the circulation is synchronized by daily return of aged neutrophils from the circulation into the BM,513 these findings implicate neutrophils as critical mediators of HSPC localization at steady state and upon enforced egress. On the molecular level, the recognition that all physiological, pathological, and pharmacological mobilization stimuli described to date interfere with VLA4 or CXCR4 signaling or both (Figure 1) highlights the key roles of these two pathways in HSPC trafficking.

Open questions and future directions

Homeostatic HSPC trafficking

The physiological function and regulation of daily HSPC egress remain elusive. We know that, similar to their mature counterparts, the release of HSPCs from the BM follows a circadian
rhythm, with sympathetic nervous system-derived adrenergic signals acting through beta(3)-adrenergic receptors on BM stroma to downregulate CXCL12 signaling and (therefore retention). While we understand the purpose of migration of more differentiated hematopoietic cells out of the BM to undergo maturation, encounter antigens, proliferate, etc., the role(s) of HSPCs found in blood and other peripheral tissues is speculative. Exchange between different parts of the hematopoietic system has been suggested to be mediated by homeostatic HSPC migration and is supported by observations from parabiotic as well as partially irradiated mice. An alternative explanation implies a possible immune surveillance function of HSPCs that have also been found in the lymphoid system and non-hematopoietic tissues with crucial immune functions such as the intestine. Furthermore, expression of MHC class II molecules, otherwise restricted to professional antigen-presenting cells, has been detected in HSPCs.

**Prediction of HSPC mobilization success and failure**

With regard to inadequate HSPC mobilization, one must distinguish between disease- and/or treatment-associated failure and failure of G-CSF mobilization in healthy donors. Of note, less than 1% of “healthy allogeneic donors” fail to collect an optimal (5×10⁶ CD34(+) or minimal (2×10⁶ CD34(+) amount of CD34+ cells after a standard 4–5-day regimen of G-CSF mobilization. This relatively uncommon event may represent the extreme heterogeneity of HSPC mobilization seen among the general population with no known medical conditions or prior exposure to chemotherapy or radiation. Thus, CD34 counts between 5 and 500 cells per µl blood have been reported. The general consensus, derived from studies of poor- and well-mobilizing mouse strains, as well as repetitive mobilization of healthy donors, is that genetic factors determine the mobilization response in healthy individuals. However, single nucleotide polymorphisms in any of the obvious candidate genes (including CXCL12, VCAM1, and CD44) do not correlate with mobilization efficacy in response to G-CSF or plerixafor in larger population studies. Knowledge of high or low HSPC mobilization potential could be translated into donor screening prior to mobilization to help predict response and potentially to guide the best mobilization strategy.

The probability of mobilization failure in patients directly correlates with the amount and extent of prior cytotoxic exposure. Other clinical and demographic features of normal donors and patients undergoing autologous stem cell mobilization that predict poor mobilization include diabetes mellitus, a history of diabetes mellitus, and a history of stromal cell recruitment would not allow for major changes of the cell population, comprising of different HSPC subsets rather than representing a homogenous population with an overall altered expression profile depending on the agent used. Moreover, we anticipate that concurrent analysis of peripheral blood and BM HSPC compartments will shed new light on the unique identity and specific origin of mobilized cells that respond to specific mobilizing agents. In general, single cell characterization of mobilized blood/HSPCs is expected to be particularly informative with an HSPC profile closely related to BM-resident HSPCs.

**Profiling of differentially mobilized blood**

Gene expression profiling of the HSPC populations mobilized with different agents has been performed, mostly using microarrays. Not unexpectedly, increased relative expression of genes involved in lymphoid development were detected in grafts mobilized with a CXCR4 antagonist alone and in conjunction with G-CSF as compared to G-CSF alone. A closer similarity between BM-resident and CXCR4 antagonist-mobilized HSPCs as compared to BM-resident versus G-CSF-mobilized HSPCs had been proposed to be due to the fast kinetics of CXCR4 antagonist-induced mobilization. However, the opposite was detected when comparing the three, with BM-resident HSPCs showing a profile much closer to that of G-CSF-mobilized HSPCs. It would appear from these studies that CXCR4 disruption recruits a specific rather than representative fraction of the BM HSPCs. By contrast, VLA4 antagonist + tGro-β-mobilized HSPCs have a profile very similar to that of BM-resident as well as G-CSF-mobilized HSPCs, indicating that rapid kinetics of mobilization can indeed be associated with an HSPC profile closely related to BM-resident HSPCs.

Single cell RNA sequencing (scRNA-seq) is currently revolutionizing the field of hematopoietic cancer research by defining the heterogeneity of malignant cells and the supporting network of non-malignant cells. Naturally, scRNA-seq analysis and comparison of differentially mobilized HSPCs will provide key insights and may strengthen the notion that they are comprised of different HSPC subsets rather than representing a homogenous population with an overall altered expression profile depending on the agent used. Moreover, we anticipate that concurrent analysis of peripheral blood and BM HSPC compartments will shed new light on the unique identity and specific origin of mobilized cells that respond to specific mobilizing agents. In general, single cell characterization of mobilized blood/HSPCs is expected to be particularly informative with rapid-mobilizing agents, where the kinetics of the cell recruitment would not allow for major changes of the cell identity or localization prior to BM egress.

Interestingly, despite the more elaborate isolation process for non-hematopoietic as opposed to hematopoietic cells, a detailed characterization of the stromal populations using single cell approaches has been published already. However, the contribution of the newly identified populations to HSPC retention remains unexplored. Ultimately, simultaneous analysis of stroma and HSPCs based on their proximity using spatial transcriptomics promises to reveal potentially unique relationships between certain stromal and hematopoietic cell types and thereby define the biological roles of the long-argued diversity within the hematopoietic niche.
Summary
Pharmacologically induced egress of HSPCs from the BM has become an indispensable tool in HSCT with all autologous and over 80% of allogeneic transplants performed with mobilized blood. Mechanistic insights gained from studying the complex chain of events induced during mobilization with G-CSF have paved the way for the rational design of alternative mobilization approaches without the inherent shortcomings of G-CSF such as slow mode of action and side effects. Similar to the recruitment of healthy HSPCs into the circulation, targeting of various BM retention pathways has been explored as a means to sensitize leukemic cells and thereby improve the efficacy of chemotherapy. Moreover, mobilization of HSPCs as a non-cytotoxic conditioning strategy as well as for gene therapy represents two other applications of mobilization beyond mere collection of a stem cell graft. Understanding the physiological function of homeostatic HSPC trafficking and identifying the genetic determinants of mobilization efficiency, along with characterizing the differentially mobilized HSPC populations on a single cell level, represent some of the directions of future mobilization research.

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