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***Abstract*:** Hematopoietic stem-cell transplantation (HCT) and stem-cell–based gene therapies rely on the ability to collect sufficient CD34+ hematopoietic stem and progenitor cells (HSPCs), typically *via* peripheral blood mobilization. Commonly used HSPC mobilization regimens include single-agent granulocyte colony-stimulating factor (G-CSF), plerixafor, chemotherapy, or a combination of these agents. These regimens, however, frequently require multiple days of injections and leukapheresis procedures to collect adequate HSPCs for HCT (minimum = $>2 \times 10^6$ CD34+ cells/kg; optimal = $5–6 \times 10^6$ CD34+ cells/kg). In addition, these regimens frequently yield suboptimal CD34+ HSPC numbers for HSPC-based gene-edited therapies, given the significantly higher HSPC number needed for successful gene-editing and manufacturing. Meanwhile, G-CSF is associated with common adverse events such as bone pain as well as an increased risk of rare but potentially life-threatening splenic rupture. Moreover, G-CSF is unsafe in patients with sickle-cell disease, a key patient population that may benefit from autologous HSPC-based gene-edited therapies, where it has been associated with unacceptable rates of serious vaso-occlusive and thrombotic events. Motixafortide is a novel CXCR4 inhibitor with extended *in vivo* activity (>48 h) that has been shown in preclinical and clinical trials to rapidly mobilize robust numbers of HSPCs in preparation for HCT, while preferentially mobilizing increased numbers of more primitive HSPCs by immunophenotyping and single-cell RNA expression profiling. In this review, we present a history of stem-cell mobilization and update of recent innovations in novel mobilization strategies with a specific focus on the development of motixafortide, a long-acting CXCR4 inhibitor, as a novel HSPC mobilizing agent.

Keywords: CXCR4 inhibition, G-CSF, hematopoietic stem-cell mobilization, hematopoietic stem-cell transplantation, hematopoietic stem-cell–based gene therapy, motixafortide

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The search for hematopoietic stem cells

The pathologist Franz Ernst Christian Neumann (1834–1918) has been credited as one of the earliest scientists to theorize that the site of blood formation may reside within the bone marrow; while also proposing the theory that a single cell-type might be the origin of all blood cell lineages.[1] The scientist and pathologist Alexander A. Maximow similarly developed a theory of a common cell of origin for the complete hematopoietic system, and in 1909, further proposed the idea of microenvironmental niches in which these cells resided within the bone marrow.[2] Experimental evidence in support of these theories, however, would remain elusive throughout the first half of the 20th Century.

In 1945, the civilian populations in Hiroshima and Nagasaki surviving the initial atomic bomb explosions were exposed to high doses of radiation, leading to clinical descriptions of a 'radiation syndrome' characterized by recurrent

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infections, bleeding complications, and mortality that we now understand were related to radiation-induced hematopoietic failure.[3] Several subsequent studies that replicated radiation syndrome in mice showed that hematopoietic failure could be prevented by shielding the spleen or femur with lead as well as by intravenous (IV) infusion of spleen or marrow cells into mice to rescue them from lethal irradiation.[4] These studies represent some of the earliest experimental evidence that the bone marrow and spleen (in mice) contained essential hematopoietic progenitors and that bone marrow infusion may represent an effective therapeutic modality. In 1957, E. Donnall Thomas and colleagues published early reports on the safety of allogeneic bone marrow infusions administered in conjunction with lethal doses of radiation or high-dose chemotherapy in a series of patients with chronic myeloid leukemia, multiple myeloma (MM), ovarian cancer, cancer of unknown primary, and a comatose patient following massive intracranial hemorrhage.[5] In this report, they made a number of important observations, including the striking conclusion that 'the definition of an adult dose of marrow in the sense of the amount needed to produce repopulation of the marrow space of an adult man after lethal radiation would be helpful'.[5] This conclusion underscored the fact that at the time there was no definitive experimental evidence documenting the existence of a single multipotent hematopoietic stem cell (HSC) capable of complete hematopoietic reconstitution. Fortunately, the first experimental proof of HSCs would be reported just a few years later in 1961 in a series of breakthrough publications from Till, McCulloch and colleagues, including an experiment in which they induced clonal markers in donor marrow by sublethal irradiation, and then plated cells in numbers that made visible colonies at day 10.[6] They observed that each colony shared a common chromosomal marker that was distinctive and that this clonal marker existed in all dividing cells of the colony, thus proving definitively that the colony-forming unit (CFU) was a single clonogenic cell. Therefore, HSCs came to be defined functionally as single cells with the capability of long-term self-renewal and multipotency.

Throughout the 1970s and 1980s, experimental and clinical testing of HSCs continued to be defined by the functional CFU assay. Furthermore, it still remained unknown what the contribution of each different hematopoietic stem and progenitor cell (HSPC) subset was to complete hematopoietic reconstitution. Consequently, stem-cell biologists were left to debate whether long-term self-renewal was a property of a single primitive HSC or perhaps whether many different HSPC subsets were capable of long-term self-renewal, making a diverse repertoire of HSPCs a necessary feature of clinical 'stem-cell' grafts. To address this question, beginning in the 1980s, several groups developed monoclonal antibodies (mAbs) and conducted fluorescence-activated cell sorting (FACS)-based HSPC profiling to prospectively isolate HSPCs and experimentally test each HSPC population for both multipotency and self-renewal using murine *in vivo* stem-cell transplant and secondary transplant reconstitution models.[7,8] Importantly, much of this pioneering work was first described in mice. One particularly notable advance in humans, however, was achieved by Civin and colleagues, when they reported that leukemia cell lines and a relatively small proportion of normal bone marrow cells expressed a surface protein named My10, a marker we now call CD34.[9] They further observed by CFU assay that CD34+ normal bone marrow cells were capable of both long-term self-renewal and broad multilineage reconstitution, giving rise to the use of CD34+ as a surface marker for quantifying human HSPC numbers and establishing CD34+ cell number as the gold-standard for dosing human HSPCs for hematopoietic stem-cell transplantation (HCT) still used to this day.

Development of peripheral blood HSPC mobilization regimens

Throughout the 1970s and early 1980s, clinical development of bone marrow transplantation also advanced. Relatively few HCTs, however, were performed throughout the 1980s, averaging <500 HCTs/year in the United States by 1989.[10] Moreover, virtually all HCTs at that time were performed using bone marrow as a source of HSPCs, which required HSPC donors to undergo numerous bone marrow aspirations under general anesthesia in a surgical operating room in order to collect sufficient HSPCs for HCT. Therefore, a number of logistical challenges and risks associated with this approach led the field to pursue alternative sources of HSPCs. Meanwhile, low

levels of HSPCs circulating in the peripheral blood (PB) had been described but were present at such low levels that they were felt not to be clinically useful.[11] In 1976, Richman and colleagues, however, reported that administration of chemotherapy markedly increased the number of PB HSPCs, as assessed by CFU, suggesting that clinically significant numbers of HSPCs were capable of being mobilized to the PB.[12] This observation led to a number of subsequent studies developing PB HSPC mobilization regimens and ultimately to Food and Drug Administration (FDA) approvals for the use of granulocyte colony-stimulating factor (G-CSF) alone or in combination with chemotherapy or plerixafor for PB HSPC mobilization. The ability to reliably collect PB HSPCs as a clinically viable HSPC source for HCT along with a number of other developments in the field of HCT contributed significantly to a steady increase in the annual number of both autologous (auto) and allogeneic (allo) HCTs performed in the United States over the ensuing decades. As of 2019, more than 14,000 auto-HCTs and nearly 10,000 allo-HCTs were performed in the United States alone, with virtually all auto-HCTs and ~70% of allo-HCTs now being performed using PB HSPCs.[10]

G-CSF and HSPC mobilization

During the 1980s, a number of pivotal studies led to the discovery of hematopoietic growth factors, including G-CSF, and characterized the critical role these factors play in regulating bone marrow HSPCs.[13,14] By 1986, G-CSF had been cloned, thus enabling its development as a therapeutic agent. A number of studies that followed demonstrated significant improvement in the number of PB-mobilized HSPCs able to be collected for auto-HCT following high-dose chemotherapy in combination with G-CSF, leading to G-CSF-based PB HSPC mobilization regimens becoming widely adopted.[15,16] Meanwhile, the exact mechanism G-CSF-mediated HSPC mobilization remained poorly understood. Continuing through the 1990s and 2000s, G-CSF with or without chemotherapy remained the most commonly used HSPC mobilizing regimen. Despite multiple days of injections and up to 4 or more apheresis procedures, however, randomized controlled trials demonstrated that 10–30% of patients undergoing auto-HCT for non-Hodgkin's lymphoma (NHL) or MM remained unable to mobilize the minimum number of 2×10^6 CD34+ cells/kg necessary for reliable engraftment.[17] Furthermore, 30–60% of patients remained unable to collect the optimal number of 5–6 million CD34+ cells/kg for auto-HCT.[17] Therefore, improved PB HSPC mobilization regimens were needed.

CXCR4 inhibition and HSPC mobilization

Importantly, in the early 2000s, it was reported that G-CSF induces stem-cell mobilization through two general mechanisms. The first involves the direct downregulation of stromal cell-derived factor 1 (SDF-1) (the ligand for CXCR4) in the bone marrow microenvironment *via* a direct toxic effect on bone marrow osteoblasts and transcriptional repression of SDF-1 (CXCL12) mRNA in the bone marrow stromal cells and osteoblasts.[18–20] The second mechanism involved the upregulation of proteolytic enzymes such as neutrophil elastase from neutrophils within the bone marrow, ultimately leading to *in situ* cleavage of multiple tethers that function to retain HSCs within the hematopoietic bone marrow niche. One of these tethers includes SDF-1, which when proteolytically cleaved leads to decreased CXCR4/SDF-1 signaling and enables egress of HSPCs from the bone marrow to the PB.[21,22] Based on these mechanisms, a number of approaches to block CXCR4 were developed, and subsequent studies confirmed the pivotal role of the CXCL12/CXCR4 ligand-receptor axis in HSPC localization to the bone marrow and mobilization into the PB (Figure 1).[23]

In 2005, a small molecule bicyclam inhibitor of CXCR4 (AMD3100, plerixafor) was reported to result in rapid mobilization of both murine and human HSPCs to PB, with peak mobilization of HSPCs occurring between 2 and 4 h in mice and 6 and 9 h in humans.[23] In contrast, the peak mobilization of HSPCs with G-CSF alone occurs between 4 and 6 days.[24] The combination of G-CSF followed by CXCR4 blockade with plerixafor synergized to significantly increase HSPC mobilization compared with either agent alone, however.[23] Therefore, in 2009, DiPersio and colleagues reported the results of two large, international, randomized, controlled pivotal phase III trials, which showed that blockade of SDF-1 binding to CXCR4 with plerixafor in combination with G-CSF enabled a significantly higher proportion of patients to meet

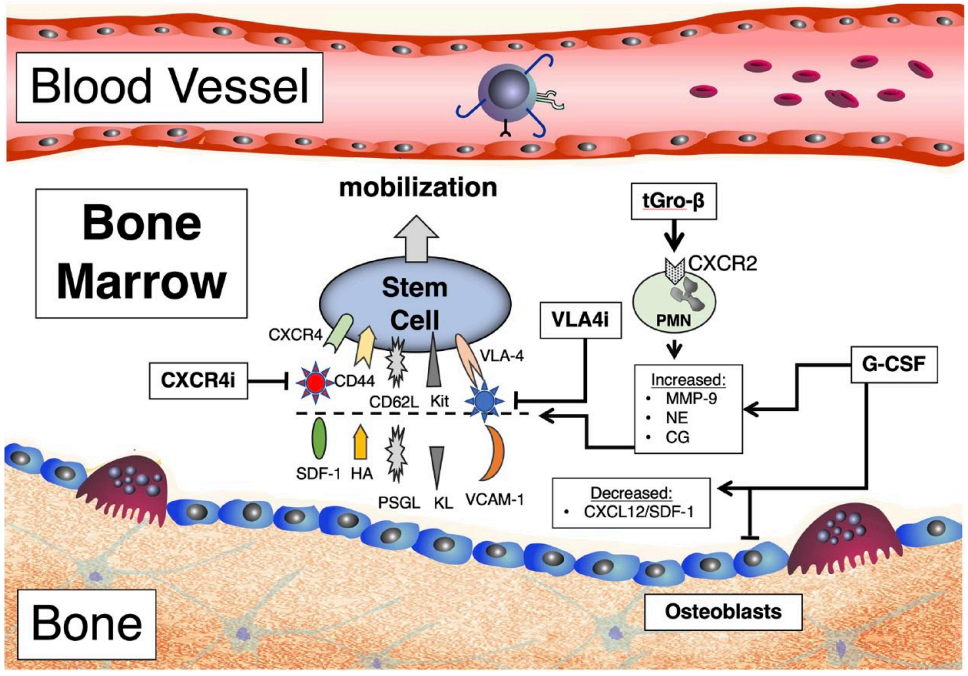

Figure 1. The bone marrow niche and stem-cell mobilization pathways.
A schema of the bone marrow niche and relevant pathways involved in regulating HSPC retention within the bone marrow and mobilization to the peripheral blood.

their primary HSPC collection goals prior to auto-HCT for MM and NHL, when compared with G-CSF alone.[25,26] Nevertheless, while representing the most effective HSPC mobilization regimen available, as many as 15–35% of patients still remained unable to meet collection goals despite receiving up to four injections of plerixafor, eight injections of G-CSF, and undergoing four leukapheresis procedures.[25,26] Furthermore, the proportion of patients who are unable to meet optimal collection goals has increased over the past decade, with recent data suggesting that patients undergoing HCT in the current era have multiple risk factors for impaired HSPC mobilization. For example, increasing age of patients undergoing auto-HCT for MM is a strong risk factor for poor mobilization, with the proportion of patients ⩾65 years of age undergoing HCT in the United States increasing from 11% in 2000 to 22% in 2009 and to 36% in 2019.[10,27,28] In addition, standard induction therapy for MM, which is the most common indication for autologous HCT in the United States, now includes 3-drug [immunomodulatory imide drug (IMiD), proteasome inhibitor (PI), and glucocorticoid] and 4-drug induction regimens (IMiD, PI, glucocorticoid, and an anti-CD38 mAb).[29,30] Both of these induction regimens prior to stem-cell mobilization are associated with reduced HSPC yields.

Therefore, a significant unmet need remains to further improve the success of HSPC mobilization in order to increase access to HCT for patients who might otherwise fail to mobilize the minimum number of CD34+ cells/kg; to increase the proportion of patients able to mobilize optimal numbers of CD34+ cells/kg ensuring rapid and consistent multilineage engraftment; and to reduce healthcare resource utilization by reducing the number of injections and apheresis days needed for patients to meet collection goals.

Extended CXCR4 inhibition with motixafortide and HSPC mobilization

Preclinical and clinical data have demonstrated that CXCR4 expression varies across CD34+ HSPC subsets, with some of the highest levels of CXCR4 expression on lineage-committed CD34+ plasmacytoid dendritic cell precursors and relatively lower levels of CXCR4 expression

on more primitive CD34+ HSCs and multipotent progenitors (MPPs).[31] Furthermore, the use of plerixafor, a relatively low-affinity (Ki: 652 nM), short-acting CXCR4i, has been shown to mobilize HSPC grafts with unique CD34+ subsets compared with G-CSF, including higher proportions of lineage-committed CD34+ progenitors, mature leukocytes, and lymphocytes.[31,32] These studies suggest that strongly CXCR4+ lineage-committed HSPC subsets and maturing leukocytes mobilize rapidly to PB in the presence of relatively transient CXCR blockade. These studies also suggest that optimizing CXCR4 blockade with more robust or longer acting CXCR inhibition may increase CD34+ HSPC mobilization effectiveness while mobilizing differential HSPC subsets, including primitive CD34+ HSCs and MPPs with lower baseline levels of CXCR4 expression.[31]

Motixafortide (BL-8040, BKT140) is a novel, synthetic, cyclic-peptide that functions as a selective antagonist of CXCR4, with a high CXCR4-binding affinity (Ki: 0.32 nM) and long receptor occupancy time resulting in extended clinical activity lasting >48 h following a single subcutaneous injection.[33–35] In preclinical mouse studies, a single injection of motixafortide resulted in rapid and robust HSPC mobilization to PB within 0.5–2 h after injection as well as a dose-dependent increase in mobilization of monocytes, B-cells, and T-cells.[36,37] Meanwhile, motixafortide mobilized significantly higher numbers of HSPCs (7.1-fold over control) when compared with plerixafor alone (4.2-fold over control) ($p < 0.05$). When tested in combination, motixafortide plus G-CSF resulted in a synergistic increase in mobilized HSPCs to PB (76.8-fold over control), which was significantly higher than plerixafor plus G-CSF (46.4-fold) ($p = 0.001$).[36] Subsequent studies demonstrated that 100% of mice transplanted with motixafortide-mobilized HSPCs maintained engraftment for >4 months, as compared with only 73% of mice transplanted with plerixafor-mobilized HSPCs. Furthermore, these HSPCs were capable of successfully re-establishing complete hematopoiesis in 100% of mice on serial re-transplant in lethally irradiated and secondarily transplanted mice, indicating that BL-8040-mobilized HSPCs contain high numbers of primitive, multipotent HSCs capable of long-term self-renewal, and robust hematopoietic reconstitution.[36]

Motixafortide HSPC mobilization in healthy volunteers

Motixafortide was subsequently tested for safety in humans in a two-part, phase I study (NCT02073019), administered *via* subcutaneous injection to healthy subjects. In part 1 of the study, a total of 25 healthy volunteers were enrolled and treated with up to 2 injections of placebo or a dose-escalation of motixafortide at 0.5, 0.75, and 1 mg/kg. At all dose levels, motixafortide was determined to be safe and well-tolerated with predominately low-grade injection site reactions occurring in a total of 88% of subjects, including 50% of patients receiving placebo. Following the first-dose administration of motixafortide, PB CD34+ counts rapidly increased reaching maximal levels within 2–4 h of the first dose. By 24 h, PB CD34+ levels had only slightly declined but remained five- to seven-fold higher than baseline. Upon administration of the second dose, an additional increase in CD34+ was observed with sustained PB CD34+ HSPCs and then a slow decline reaching baseline ~48 h after injection, consistent with rapid, robust PB HSPC mobilization and extended *in vivo* activity. Part 2 of the study was an open-label dose expansion study that enrolled an additional eight healthy volunteers who each received a single injection of motixafortide at 1 mg/kg followed by a leukapheresis procedure starting 4 h after injection. At the 1 mg/kg dose level, motixafortide was again found to be safe and well-tolerated with predominately low-grade injection site reactions occurring in 100% of subjects, while enabling collection of a median of 11.2×10^6 CD34+ cells/kg in a single leukapheresis. These promising early phase clinical data documenting the safety and efficacy of motixafortide in healthy volunteers thus served as the basis for further clinical development of motixafortide as an HSPC mobilization agent in subsequent clinical trials.

Motixafortide plus G-CSF HSPC mobilization for auto-HCT

The first study in humans using motixafortide in combination with G-CSF for HSPC mobilization was conducted as a single-arm, open-label, single administration, dose-escalation, phase I study (NCT01010880). In this study, a total of 18 MM patients underwent standard HSPC mobilization with chemotherapy (cyclophosphamide) plus G-CSF prior to auto-HCT. Following

administration of chemotherapy and G-CSF over a 10-day mobilization protocol, patients then received a single subcutaneous injection of motixafortide in escalating dose cohorts starting with 0.006 mg/kg in dose-level 1 and increasing to 0.9 mg/kg in dose-level 5. In this study, motixafortide in combination with G-CSF and chemotherapy was safe and well-tolerated, with the majority of adverse events (AEs) reported occurring during the chemotherapy and G-CSF period of mobilization. Whereas only 34.4% of treatment emergent adverse events (TEAEs) occurred after motixafortide administration, with all such TEAEs being graded as mild (76.9%) to moderate (23.1%) in severity. Meanwhile, motixafortide at the higher dose levels of 0.3–0.9 mg/kg when added to standard mobilization resulted in accelerated mobilization of HSCs and enabled an increased number of patients to reach their collection goal in a single leukapheresis. Furthermore, 100% of patients who ultimately underwent auto-HCT experienced durable multilineage engraftment.

The GENESIS clinical trial

To confirm these early phase clinical trial results, a two-part, phase III study was performed to evaluate the safety and efficacy of motixafortide in combination with G-CSF to mobilize HSPCs in MM patients undergoing auto-HCT (NCT03246529). Part 1 of the study was a single-center, open-label, lead-in design, with each patient receiving motixafortide and G-CSF mobilization. Part 2 of the study was an international, randomized, placebo-controlled, double-blinded design, in which patients were randomized 2:1 to motixafortide plus G-CSF or placebo plus G-CSF. The mobilization protocol for both part 1 and part 2 involved patients receiving G-CSF (10 mcg/kg, subcutaneous) on the morning of days 1–5, and days 6–8 if needed; motixafortide (1.25 mg/kg, subcutaneous) or placebo on the evening of day 4, and day 6 if needed; and starting leukapheresis on the morning of day 5. The primary endpoint was the proportion of patients collecting $\geq 6 \times 10^6$ CD34+ cells/kg in up to two apheresis sessions.

In part 1 of the GENESIS study, a total of 11 patients were enrolled, with 82% (9/11) meeting the primary endpoint of collecting $\geq 6 \times 10^6$ CD34+ cells/kg in up to 2 apheresis sessions and 64% (7/11) collecting to goal in just 1 leukapheresis procedure. Notably, 100% (11/11) of patients successfully met the collection goal within 4 leukapheresis procedures, with a median of 9×10^6 CD34+ cells/kg collected among all patients in part 1. The most common AEs reported were local injection site reactions occurring in 91% (10/11) of patients. Based on these data and prespecified safety and efficacy endpoints for part 1 of the study, the independent data monitoring committee recommended proceeding to part 2 of the study.[38]

In part 2 of the study, a total of 122 patients from 18 sites in 5 countries were enrolled, with 92.5% of patients mobilized with motixafortide plus G-CSF meeting the primary endpoint compared with 26.2% with placebo plus G-CSF [odds ratio (OR) = 53.3, $p < 0.0001$]. Meanwhile, 88.8% of patients mobilized with motixafortide plus G-CSF met the collection goal in 1 leukapheresis procedure compared with only 9.5% with placebo plus G-CSF (OR = 118.0, $p < 0.0001$). The median number of CD34+ HSPCs collected in one leukapheresis procedure by a single injection of motixafortide added to G-CSF was 10.8×10^6 CD34+ cells/kg compared with 2.25×10^6 CD34+ cells/kg with placebo plus G-CSF. Meanwhile, the actual number of CD34+ HSPCs infused for auto-HCT was $<6 \times 10^6$ CD34+ cells/kg in both treatment arms with the cell dose infused being at the discretion of the treating physician, according to local practice. Time to engraftment of both neutrophils and platelets, graft durability, progression free survival, and overall survival were comparable between the two cohorts. Motixafortide plus G-CSF was observed to be safe and well-tolerated when compared with placebo plus G-CSF, with a total 93.8% (75/80) patients experiencing any grade TEAE in the motixafortide plus G-CSF cohort compared with the 83.3% (35/42) in the placebo plus G-CSF cohort.[39]

Contemporary randomized controlled trials directly comparing the relative effectiveness of available HSPC mobilization regimens are lacking. Within the limits of cross trial comparisons, the GENESIS trial results compare favorably with the two largest randomized controlled studies published in 2009 which compared plerixafor plus G-CSF with placebo plus G-CSF in MM and NHL patients.[25,26] Across these studies, the

Table 1. Relative effectiveness of HSPC mobilizing regimens for HCT.

	Allo-HCT			MM/NHL patients for auto-HCT		
	G-CSF	**Plerixafor**	**Motixafortide**	**G-CSF + placebo**	**G-CSF + plerixafor**	**G-CSF + motixafortide**
Author(s)	Xiang J, *et al.*	Schroeder MA, *et al.*	Rettig MP, *et al.*	DiPersio JF, *et al.*; Crees ZD, *et al.*	DiPersio JF, *et al.*; DiPersio JF, *et al.*	Crees ZD, *et al.*
Study population	Allo-donor	Allo-donor	Allo-donor	MM/NHL	MM/NHL	MM
Total number of subjects[a]	1025	56	25	344	298	80
Number of injections[b]	5	1	1	5	5 + 1	5 + 1
Median HSPCs collected in first apheresis ($\times 10^6$ CD34+ cells/kg)	7.57	2.1–2.27	2.32–3.28	2.25–2.29	7.01	10.8
Citations	PMID: 34555850	PMID: 28292947	doi.org/10.1182/blood-2018-99-109701	PMID: 19720922 PMID: 19363221 PMID: 37069359	PMID: 19720922 PMID: 19363221	PMID: 37069359

G-CSF, granulocyte colony-stimulating factor; HCT, hematopoietic stem-cell transplantation; HSPC, hematopoietic stem and progenitor cell; MM, multiple myeloma; NHL, non-Hodgkin's lymphoma.
A table comparing the relative effectiveness of HSPC mobilization regimens with references to the relevant studies.
[a]Number of subjects represents the total number of patients treated with the specified mobilization regimen across all studies cited.
[b]Number of injections represents the number of injections administered prior to the first apheresis.

proportion of patients who mobilized optimal HSPC numbers ($5–6 \times 10^6$ CD34+ cells/kg) in one apheresis were 4.2–17.3% with placebo plus G-CSF, 27.9–54.2% with plerixafor plus G-CSF, and 88.8% with motixafortide plus G-CSF (Table 1).[25,26,39]

As a correlative study in conjunction with the GENESIS Trial, immunophenotypic and transcriptional profiling *via* multicolor FACS and single-cell RNA sequencing (scRNA seq) of CD34+ HSPCs from the patients mobilized on the GENESIS Trial as well as a prospectively enrolled cohort of demographically similar MM patients mobilized with plerixafor plus G-CSF and a cohort of allogeneic HSPC donors mobilized with single-agent motixafortide, plerixafor, or G-CSF alone. These studies revealed that motixafortide plus G-CSF mobilized significantly higher HSPC numbers in eight out of nine CD34+ HSPC subsets when compared with placebo plus G-CSF, including higher numbers of immunophenotypically primitive HSCs and MPPs. In addition, motixafortide plus G-CSF also mobilized significantly higher numbers of MPPs, common myeloid progenitors, and common lymphoid progenitors when compared with plerixafor plus G-CSF. Meanwhile, scRNA seq demonstrated that motixafortide-mobilized HSPCs expressed unique transcriptional profiles associated with self-renewal, regeneration, and quiescence, when compared with HSPCs mobilized with plerixafor or G-CSF.[40]

In summary, motixafortide in combination with G-CSF for HSPC mobilization prior to auto-HCT was safe and effective, enabling significantly higher numbers of patients to mobilize optimal numbers of CD34+ HSPCs with less injections and leukapheresis procedures when compared with G-CSF alone. In addition, correlative data from these studies suggest that extended CXCR4 inhibition with motixafortide leads to differential mobilization of various HSPC subsets with increased numbers of immunophenotypically primitive stem cells and MPPs which express transcriptional programs associated with enhanced self-renewal, regeneration, and quiescence.

Motixafortide HSPC mobilization for allo-HCT

Motixafortide has been evaluated as a single-agent HSPC mobilization regimen for allo-HSPC donors in a multicenter, open-label, single-arm, 2-part, phase II study (NCT02639559). In this study, a total of 25 donor-recipient pairs aged 18–70 years were enrolled and mobilized with a single injection of motixafortide dosed at 1.0 or 1.25 mg/kg. Donors underwent leukapheresis within 3–4 h following motixafortide injection. In this study, the primary endpoint was the proportion of donors collecting $\geq 2.0 \times 10^6$ CD34+ cells/kg within two leukapheresis procedures. Key secondary endpoints included the number of donors collecting to goal in one leukapheresis as well as safety/toxicity, engraftment kinetics, and rates of graft-*versus*-host disease (GVHD). The investigators observed that a single injection of motixafortide resulted in 92% (22/24) of allo-donors collecting $\geq 2.0 \times 10^6$ cells/kg within two leukapheresis procedures, with 100% of donors receiving the higher dose of motixafortide at 1.25 mg/kg meeting that goal. Similar to plerixafor, approximately two-thirds (16/24) of these allo-donors collected $\geq 2.0 \times 0^6$ cells/kg after a single leukapheresis procedure.[31,40] Meanwhile, motixafortide was well-tolerated with Grade 1 local injection site reactions such as pain, erythema, and edema/hives occurring in 80% (20/25) of donors. Of the 22 recipients who underwent allo-HCT with motixafortide-mobilized cells on study, engraftment kinetics were typical of that observed with PB-mobilized HSPCs using alternative regimens, with a median time to neutrophil engraftment of 13 days (range = 11–26 days) and median time to platelet engraftment of 18 days (range = 15–41 days). Rates of acute and chronic GVHD were also similar to previously reported historical rates, with a cumulative incidence of grade II–IV acute GVHD at day 180 post-HCT of 32% (7/22) and chronic GVHD at 2 years post-HCT of 60%. Notably, mild-to-moderate chronic GVHD rate occurred at a rate of 39% (7/18), with only one case of severe chronic GVHD.[40]

Contemporary randomized controlled trials directly comparing the relative effectiveness of available HSPC mobilization regimens are lacking for allo-donors. Findings of the single-arm study with motixafortide, however, are suggestive of particularly rapid HSPC mobilization of $\geq 2 \times 10^6$ CD34+ cells/kg in 92% of allo-donors with one injection and ≤2 leukapheresis procedures. By comparison, historical data with G-CSF alone indicate that up to 60% of donors required ≥5 G-CSF injections and ≥2 leukapheresis procedures.[41] Meanwhile, with plerixafor alone an estimated 34% of donors required ≥ 2 plerixafor injections and ≥ 2 leukapheresis procedures.[31] More recently, a retrospective analysis was performed of 1361 related allo-donors who underwent HCT comparing standard allo-donor mobilization with G-CSF *versus* five alternative mobilization regimens, including granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF plus GM-CSF, GM-CSF plus plerixafor, plerixafor alone, and motixafortide alone.[42] In this study, CXCR4 inhibition alone resulted in similarly rapid mobilization of HSPCs in a single day but ultimately lower overall HSPC yields were observed when compared with cytokine-based regimens administered over 5 or more days (Table 1).[42]

Additional correlative analyses performed as part of the multicenter, open-label, single-arm, two-part, phase II study in allo-donors (NCT02639559) aimed to characterize the various CD34+ HSPC subsets and T-cell subsets mobilized with motixafortide, using multicolor FACS. These analyses demonstrated that motixafortide resulted in the mobilization and collection of three distinct CD34+ HSPC populations. The first was a population of more primitive HSCs, MPPs, and common myeloid progenitors (CD45RA− $CD123^{lo}$ CD303−), which comprised 66.0% of total CD34+ HSPCs. The second was a population of granulocytic myeloid progenitors and common lymphoid progenitors (CD45RA+ $CD123^{lo}$ CD303−), which comprised 23.1% of the total CD34+ HSPCs. Finally, the authors observed a population of lineage-committed plasmacytoid dendritic cell precursors (CD45RA+ CD123+ CD303+), which comprised 10.9% of the total CD34+ HSPCs. Interestingly, the plasmacytoid dendritic cell precursors in this study have been shown to express significantly higher levels of CXCR4 and therefore appear to be preferentially mobilized with CXCR inhibitor containing regimens, a phenomenon previously observed with plerixafor as well.[31] Further immunophenotyping of T-cells mobilized with motixafortide demonstrated increased numbers of CD8+ naïve T-cells and central memory T-cells compared with CD8+ effector

T-cells and effector memory T-cells. By contrast, motixafortide resulted in two- to four-fold increased mobilization of all CD4+ T-cell subsets. In the case of T-cells, there was only a loose correlation with the level of CXCR4 expression and magnitude of fold-increase in each T-cell subset mobilized, suggesting that CXCR4 expression is but one factor that influences the T-cell component of CXCR4 inhibitor mobilized allo-donor grafts.[43]

In summary, single-agent motixafortide is capable of rapidly mobilizing PB HSPCS in allo-donors with less injections relative to G-CSF or plerixafor. CXCR4 inhibition with motixafortide or plerixafor in combination with G-CSF, however, may synergize to yield higher numbers of PB HSPCs compared with either respective CXCR4 inhibitor as a single agent. In addition, daily injections of single-agent G-CSF administered over 4–5 days appear to mobilize a higher total numbers of PB HSPCs in allo-donors, relative to a single injection of plerixafor or motixafortide alone. Meanwhile, motixafortide preferentially mobilizes primitive HSPCs, along with CD8+ naïve T-cells, central memory T-cells, and a broad repertoire CD4+ T-cell subsets.

Motixafortide and HSPC mobilization for HSPC-based gene-edited cellular therapies

Recent technological and scientific advances have facilitated the development of HSPC-based gene transduction and gene-editing therapies as potentially curative treatments for a number of hematologic diseases arising from specific genetic alterations, such as thalassemia and sickle-cell disease (SCD). As with HCT, the effectiveness of HSPC-based gene transduction and editing therapies relies, in part, on the ability to collect sufficient CD34+ cells. The numbers of HSPCs needed for such gene therapies, however, are significantly higher than what is needed for standard HCT, with typically $>10–15\times10^6$ CD34+ cells/kg needed to reliably manufacture these gene-edited cellular therapies due to a number of technical factors.[44] In addition, due to potential loss of long-term engraftment of *ex vivo* genetically manipulated HSCs following infusion, HSPC backup grafts are necessary as a safety mechanism to rescue patients in the event of such graft failure. The most effective FDA approved HSPC mobilizing regimen at this time is G-CSF in combination with plerixafor.[25,26] Yet, G-CSF is unsafe in patients with SCD due to the increased risk of life-threatening vaso-occlusive episodes. Meanwhile, non-SCD patients typically require numerous G-CSF injections and multiple leukapheresis procedures with HSPC yields that are often suboptimal.[45–47] In addition, single-agent plerixafor is a relatively weak HSPC mobilizer, requiring multiple injections and leukapheresis procedures while also yielding suboptimal HSPC numbers for gene-edited therapies. Recent data highlight this issue, reporting that the majority of SCD patients mobilized with plerixafor alone remained unable to collect $\geqslant5\times10^6$ CD34+ cells/kg after two mobilization and collection cycles, making this potentially curative therapy inaccessible to a large number of otherwise eligible SCD patients.[44] Therefore, the development of safe, effective and efficient 'G-CSF-free' HSPC mobilization regimens specifically for HSPC-based gene therapies remain an unmet need.

Furthermore, the therapeutic benefit of HSPC-based gene therapies also depends significantly on the ability of genetically manipulated HSPCs to stably engraft and persist *in vivo* after infusion into patients. The presence of CD34+ continues to serve as the clinical marker for HSPCs, as it has for decades. Yet, previous work pioneered by Weissman and others in the 1980s and 1990s using FACS-based immunophenotypic profiling of HSPCs has established that CD34+ cells are highly heterogeneous, ranging from primitive HSCs (lin−, THY1+ [CD90+], CD45−, CD38−, and CD49F+) capable of long-term self-renewal and broad multilineage hematopoietic engraftment to more differentiated and lineage committed progenitor cells.[31] More recent techniques, such as scRNA seq and Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-seq), have confirmed this observation of CD34+ heterogeneity, while also contributing significantly to our current understanding of how HSPC graft source (bone marrow *versus* PB mobilization) and mobilization regimen (G-CSF *versus* plerixafor *versus* motixafortide) impact HSPC transcriptional subsets.[40,48,49] These studies underscore the need for additional research into what constitutes an 'ideal' HSPC graft for lentiviral transduction and gene-editing, as well as how CD34+ HSPC graft composition impacts HSPC manufacturing success and clinical outcomes. G-CSF-free HSPC

regimens that are not only capable of safely and effectively increasing CD34+ yields but also capable of enriching the CD34+ graft with higher-quality HSCs may be advantageous.

Novel mobilizing platforms

A number of promising novel targets and approaches for G-CSF-free mobilizing regimens are currently being explored. These include the development of novel CXCR4 inhibitors such as motixafortide, targeting the CXCR2/Groβ pathway using novel CXCR2 agonists, and use of inhibitors of the VLA-4/VCAM-1 axis. A truncated Groβ, MGTA-145, is being tested in the clinic to mobilize HSPCs from MM patients for auto-HCT (NCT04552743) and from sickle-cell anemia patients for potential gene therapy (NCT05445128). Based on the critical role of the VLA-4–VCAM-1 axis on stem-cell trafficking, the authors and others are also developing novel small molecule inhibitors of VLA-4 such as Ava4746 (an orally bioavailable alpha-4 integrin inhibitor developed by Aviara) and natalizumab (an mAb to alpha-4 integrin) as HSPC mobilizing agents when given alone or in combination with CXCR4 antagonists or CXCR2 agonists (Figure 1).[50–53]

Conclusion

Therapeutic uses for HSPCs have greatly expanded since their discovery, with PB HSPCs now representing the predominant source of HSPCs for both HCT and HSC-based gene therapies. Currently approved regimens have enhanced the number and quality of HSPCs able to be collected from the PB. These regimens, however, continue to require numerous injections and multiple apheresis days to collect sufficient cells for HCT and frequently yield suboptimal HSC numbers for lentiviral gene transduction and gene-editing. Motixafortide represents a high-affinity, long-acting CXCR4i that has been shown to be highly effective in mobilizing >95% of patients with one injection of motixafortide in combination with G-CSF for auto-HCT. In addition, >90% of allo-donors were able to collect sufficient numbers of HSPCs for allo-HCT following one injection of motixafortide as a single-agent without use of G-CSF. Ongoing studies are actively evaluating motixafortide alone and in combination with other novel mobilizing agents, with the goal of developing safe and effective, G-CSF-free, rapid mobilization regimens for HCT and HSC-based gene therapies. Relevant and ongoing correlative work in the field continues to define the various HSPC subsets mobilized by such novel HSPC mobilization regimens through immunophenotypic, scRNA seq, and multiomics approaches, in order to further understand the impact of each regimen on the HSPC graft composition and associated clinical outcomes.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Author contributions

Zachary D. Crees: Conceptualization; Writing – original draft; Writing – review & editing.

Michael P. Rettig: Conceptualization; Writing – review & editing.

John F. DiPersio: Conceptualization; Writing – review & editing

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