CHAPTER THREE

CXCR4 and Mobilization of Hematopoietic Precursors

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Contents

1. Introduction 58
2. HSPC Mobilizing Agents that Target the CXCL12/CXCR4 Axis 59
3. Donor Selection for HSPC Mobilization 64
    3.1. Selection of mice for HSPC mobilization 64
    3.2. Selection of humans for HSPC mobilization 64
4. Flow Cytometric Enumeration of Mobilized HSPCs 65
    4.1. Flow cytometric enumeration of murine HSPCs 65
    4.2. Flow cytometric enumeration of human HSPCs 65
5. Dosing and Kinetics of HSPC Mobilization by G-CSF and Plerixafor 66
    5.1. G-CSF 66
    5.2. Plerixafor 67
6. Flow Cytometric Analysis of CXCR4 Expression on Human CD34$^+$ Subsets 69
    6.1. Evaluation of cell surface CXCR4 on human CD34$^+$ cell subsets 70
7. Functional Characterization of Mobilized HSPCs 73
    7.1. *In vitro* assays of differentiation 73
    7.2. Transmigration assays 73
    7.3. Transplantation assays for mouse and human HSPCs 74
8. Concluding Remarks 80
Acknowledgment 80
References 80

Abstract

The binding of the chemokine [C-X-C motif] ligand 12 (CXCL12 or stromal cell–derived factor 1α [SDF-1α]) constitutively produced by bone marrow stromal cells and osteoblasts, to the CXC receptor (CXCR) 4, a transmembrane chemokine receptor expressed on hematopoietic stem and progenitor cells (HSPCs),

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has emerged as a key signal for HSPC trafficking to and from the bone marrow. Disruption of CXCL12/CXCR4 signaling causes leukocytosis, with the release of HSPCs, neutrophils, and lymphocytes into the peripheral blood. Although mobilized peripheral blood has become the preferred source of stem cells for both autologous and allogeneic transplantation, the optimum strategy for obtaining mobilized products from donors is the subject of ongoing study. Granulocyte colony-stimulating factor (G-CSF) and plerixafor (AMD3100) are two agents used clinically to induce HSPC mobilization by disruption of the CXCL12/CXCR4 interaction. This chapter describes current procedures used to phenotypically and functionally characterize murine and human HSPCs mobilized by G-CSF or plerixafor.

1. Introduction

The majority of hematopoietic stem and progenitor cells (HSPCs) reside in the bone marrow in a highly organized microenvironment consisting of marrow stromal cells, osteoblasts, osteoclasts, and other extracellular matrix proteins (e.g., collagens, fibronectins, proteoglycans) (Adams and Scadden, 2006; Kiger *et al.*, 2000; Kollet *et al.*, 2007; Wilson and Trumpp, 2006; Xie and Spradling, 2000). HSPCs express a number of cell surface molecules such as lymphocyte function-associated antigen-1 (LFA-1), very late antigen 4 (VLA-4), CXCR4, CXCR2, CD44, CD62L, and CD117 (c-kit) that mediate their adherence in the bone marrow (BM) microenvironment (Adams and Scadden, 2006; Lapidot *et al.*, 2005; Wilson and Trumpp, 2006). These interactions play important roles in regulating HSPC trafficking, as well as self-renewal, proliferation, and differentiation processes (Kiel and Morrison, 2008; Wilson and Trumpp, 2006).

Under normal conditions, a small number of HSPCs circulate in the peripheral blood. However, the number of circulating HSPCs can be increased 10- to 100-fold with administration of chemotherapy and/or cytokines in a process termed "stem cell mobilization" (Bensinger *et al.*, 2009; Papayannopoulou and Scadden, 2008; Winkler and Levesque, 2006). Mobilized HSPCs can be collected by large-volume apheresis techniques in numbers sufficient for use in hematopoietic stem cell transplants, and upon reinfusion, are capable of homing to the BM cavity and regenerating the full array of hematopoietic lineages. Compared to BM, use of peripheral blood stem cells in hematopoietic stem cell transplantation results in more rapid hematologic reconstitution, reduced hospitalization costs, and avoids the risks of general anesthesia and discomfort with a BM harvest (Group, 2005). Because of these advantages, the use of mobilized HSPCs over marrow as a stem cell source continues to increase such that greater than 95% of

autologous transplants and 75% of allogeneic hematopoietic stem cell transplants in adults are currently being performed with mobilized HSPCs (Bensinger *et al.*, 2009).

The optimal method for mobilization of HSPCs remains a subject of investigation. Although various agents have been used to mobilize HSPCs, only granulocyte colony-stimulating factor (G-CSF) (filgrastim, Neupogen®, Amgen, Thousand Oaks, CA), granulocyte-macrophage colony-stimulating factor (GM-CSF) (sargramostim, Leukine®, Bayer Healthcare Pharmaceuticals, Seattle, WA), stem cell factor (ancestim, Stemgen®, Amgen, Thousand Oaks, CA, available in Canada and New Zealand only), and plerixafor (Mozobil, AMD3100, Genzyme Corporation, Cambridge, MA) are approved clinically for use in stem cell mobilization (Bensinger *et al.*, 2009). Currently, G-CSF is the most commonly used agent to induce HSPC mobilization. However, because optimal mobilization requires from 4 to 6 days of G-CSF administration, donors may experience significant inconvenience, including bone pain, fatigue, headache, and nausea. Furthermore, while no long-term sequelae have been confirmed with short-term G-CSF, there are reports of serious acute toxicities related to its use as well as concerns that it can induce genetic and epigenetic modifications in HSPCs (Hernandez *et al.*, 2005; Nagler *et al.*, 2004; Shapira *et al.*, 2003; Tigue *et al.*, 2007). Accordingly, a less toxic, more rapid, and yet efficient method for collection of HSPCs from donors is still required and would represent a clear advance.

2. HSPC Mobilizing Agents that Target the CXCL12/CXCR4 Axis

Targeted disruption of the interaction between CXCR4 and CXCL12 has received considerable attention since it may provide a method to efficiently and rapidly mobilize HSPCs from the BM into the periphery as well as inhibiting the metastatic process and HIV-1 infection (Burger and Peled, 2009; Grande *et al.*, 2008; Khan *et al.*, 2007; Uy *et al.*, 2008). CXCL12 is a chemokine constitutively produced at high levels in the BM by stromal cells such as osteoblasts, endothelial cells, and a subset of reticular cells (Calvi *et al.*, 2003; Dar *et al.*, 2005; Imai *et al.*, 1999; Jung *et al.*, 2006; Ponomaryov *et al.*, 2000). It is a potent chemoattractant for HSPCs and has been shown to regulate cell adhesion, survival, and cell-cycle status (Peled *et al.*, 1999; Sugiyama *et al.*, 2006; Watt and Forde, 2008). Interestingly, CXCL12 gene polymorphism has been proposed as a conditional factor for human $CD34^+$ stem cell mobilization, with the presence of the SDF1-3′A allele as a predictive factor of good $CD34^+$ cell mobilization (Benboubker

et al., 2001). More recently, a second receptor, CXCR7, was identified that binds CXCL12 with an affinity that is approximately 10-fold higher than the affinity for CXCR4 (Balabanian *et al.*, 2005a; Burns *et al.*, 2006). Although the role of CXCR7 in CXCL12-dependent chemotaxis is not fully understood, there is evidence that CXCR7 lacks intrinsic chemotactic activity toward CXCL12, and functions instead by sequestering CXCL12 and modifying CXCR4 signaling (Boldajipour *et al.*, 2008; Hartmann *et al.*, 2008; Sierro *et al.*, 2007).

CXCR4 is a member of the large family of seven transmembrane domain receptors coupled to heterotrimeric G_i proteins and functions as a coreceptor for HIV-1 cell entry (Bleul *et al.*, 1996; Feng *et al.*, 1996; Fredriksson *et al.*, 2003; Loetscher *et al.*, 1994; Oberlin *et al.*, 1996). Both CXCL12 (Bleul *et al.*, 1996; Oberlin *et al.*, 1996) and macrophage migrating inhibiting factor (MIF) (Bernhagen *et al.*, 2007) are ligands for CXCR4. The binding of CXCR4 to CXCL12 results in activation of multiple signal transduction pathways ultimately triggering chemotaxis (Busillo and Benovic, 2007; Kucia *et al.*, 2004). Targeted disruption of either CXCL12 or CXCR4 is lethal in mice, resulting in very similar developmental defects, including the failure of HSPC migration from the fetal liver to the BM, defects in lymphoid and myeloid hematopoiesis, and cerebellar dysgenesis (Ma *et al.*, 1998; Nagasawa *et al.*, 1996; Tachibana *et al.*, 1998; Zou *et al.*, 1998). Furthermore, wildtype mice transplanted with CXCR4-deficient progenitor cells have high circulating levels of HSPCs, indicating poor retention in the BM (Christopher *et al.*, 2009; Ma *et al.*, 1999). Finally, multiple preclinical and clinical studies have shown that pharmacologic interference in the axis between marrow-derived CXCL12 and CXCR4 expressed on HSPCs using various CXCR4 modulators, including antagonist, peptide agonist, and modified CXCL12 analogues stimulate HSPC mobilization in a target-dependent manner (Nervi *et al.*, 2006; Pelus, 2008).

There are three potential mechanisms to explain how CXCR4 could regulate HSPC mobilization: (1) downregulation of cell surface CXCR4 by internalization or proteolysis, (2) disruption of the CXCL12 chemokine gradient between the BM and plasma, and (3) receptor antagonism via direct blocking of the CXCR4/CXCL12 interaction (Table 3.1). Decreased expression of CXCR4 on mobilized HSPCs has been reported following administration of G-CSF (Christopher *et al.*, 2009; Dlubek *et al.*, 2006; Levesque *et al.*, 2003; Oelschlaegel *et al.*, 2007; Semerad *et al.*, 2005), a CXCL12 analogue (met-SDF-1β) (Shen *et al.*, 2001; Yang *et al.*, 1999), and CXCL12-derived peptide agonists (CTCE-0021, CTCE-0214) (Faber *et al.*, 2007; Pelus *et al.*, 2005; Zhong *et al.*, 2004). Since native CXCL12 itself downregulates CXCR4 expression but does not result in significant mobilization (Haribabu *et al.*, 1997; Orsini *et al.*, 1999;

Table 3.1 CXCR4-mediated mobilization of murine HSPC

Compound	Class	Mechanism	Dose	Route	HSPC peak mobilization	Reference
G-CSF	Growth factor	Granulocyte expansion/activation, protease release, and cleavage of adhesion molecules, downregulation of CXCL12 in osteoblasts	250 μg/kg/day x 5 days	SC	Day 5	Molineux *et al*., 1990
Pegylated G-CSF	Growth factor	Granulocyte expansion/activation, protease release and cleavage of adhesion molecules, downregulation of CXCL12 in osteoblasts	25 μg	SC	Day 3	de Haan *et al*., 2000
Plerixafor	Bicyclam	CXCR4 antagonist	5 mg/kg	SC	3–6 h	Broxmeyer *et al*., 2005
			3 mg/kg	IV	1–3 h	Ramirez *et al*., 2008
T140	Peptide	CXCR4 antagonist	5 mg/kg	SC	1–2 h	Abraham *et al*., 2007
T134	Peptide	CXCR4 antagonist	10 mg/kg	SC	1 h	Iyer *et al*., 2008
Met-SDF-1β	CXCL12 analog	CXCR4 agonist	300 μg	IV	48 h	Shen *et al*., 2001

(*continued*)

Table 3.1 (*continued*)

Compound	Class	Mechanism	Dose	Route	HSPC peak mobilization	Reference
CTCE-0021	CXCL12 peptide analog	CXCR4 agonist	25 mg/kg	SC	1 h	Pelus, *et al.*, 2005
CTCE-0214	CXCL12 peptide analog	CXCR4 agonist	75 μg	IV	4 h	Zhong *et al.*, 2004
FucS	Sulfated polysaccharide	Disruption of CXCL12 chemotactic gradient	100 mg/kg	IV	1.5 h	Sweeney *et al.*, 2002

G-CSF, granulocyte colony-stimulating factor; Met-SDF, N-terminal methionine stromal cell---derived factor; FucS, sulfated polysaccharide fucoidan; IV, intravenous; SC, subcutaneous; n.d., not determined.

Signoret *et al.*, 1997, 1998), it is generally believed that a threshold level of CXCR4 downregulation may be required for these agents to induce HSPC mobilization (Busillo and Benovic, 2007; Kucia *et al.*, 2004). Concerning the second mechanism whereby CXCR4 could modulate HSPC mobilization, studies have shown that disruption of the CXCL12 gradient between the BM and the peripheral blood by the administration of sulfated polysaccharides (Sweeney *et al.*, 2002) or adenovirus expressing CXCL12 (Hattori *et al.*, 2001) results in an increase in circulating CXCL12 and HSPC mobilization. More relevant physiologically, recent studies have shown that a key step in G-CSF–induced HSPC mobilization is loss of CXCL12 expression by osteoblasts in the BM (Christopher *et al.*, 2009; Katayama *et al.*, 2006; Semerad *et al.*, 2005). Since similar results were observed following mobilization of mice with Flt3L or stem cell factor (Christopher *et al.*, 2009), this loss of osteoblast-produced CXCL12 may represent a common pathway in cytokine-induced mobilization. Finally, several CXCR4 antagonists have been described, of which plerixafor (Broxmeyer *et al.*, 2005; Liles *et al.*, 2003; Uy *et al.*, 2008), T140 (Abraham *et al.*, 2007), and T134 (Iyer *et al.*, 2008) have been shown to rapidly mobilize HSPCs.

Additional evidence for the critical role that CXCR4 plays in leukocyte trafficking has been obtained from patients with the genetic immunodeficiency syndrome WHIM (warts, hypogammaglobulinemia, infections, myelokathexis). WHIM syndrome is a rare congenital immunodeficiency disorder characterized by susceptibility to human papilloma virus infection-induced warts, B-cell lymphopenia and hypogammaglobulinemia, chronic noncyclic neutropenia, and BM myeloid hyperplasia with apoptosis (Gorlin *et al.*, 2000; Gulino, 2003). Most cases of WHIM syndrome have been linked to autosomal dominant mutations in CXCR4, all of which truncate the C-terminal tail of CXCR4 (Balabanian *et al.*, 2005b; Gulino *et al.*, 2004; Hernandez *et al.*, 2003). Multiple studies have demonstrated that loss of the intracellular tail of CXCR4 prevents its internalization and desensitization in response to CXCL12 (Balabanian *et al.*, 2005b; Gulino *et al.*, 2004; Kawai *et al.*, 2005). This loss of homologous desensitization leads to long-lasting activation of G-proteins and sustained functional activity of the chemokine receptor as evidenced by increased chemotaxis to CXCL12, F-actin polymerization, intracellular calcium release, and endothelial adhesion (Balabanian *et al.*, 2005b, 2008; Gulino *et al.*, 2004). Since CXCL12 is expressed constitutively at high levels in the BM, it is not surprising that WHIM leukocytes preferentially traffic to the marrow. In fact, expression of the WHIM-type mutated CXCR4 in healthy human $CD34^{+}$ cells enhances their chemotactic response to CXCL12 and BM engraftment in immunodeficient mice (Kawai *et al.*, 2007).

3. Donor Selection for HSPC Mobilization

3.1. Selection of mice for HSPC mobilization

Mice aged 8 weeks or older are used for mobilization experiments. When purchased from commercial vendors or obtained from outside sources, we allow the mice to acclimate to our facility for at least 1 week before use. All animal use should be in accordance with the guidelines of each individual's Institutional Animal Care and Use Committee, the Federal Animal Welfare Act, and conform to recommendations in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996).

Similar to humans, there is a wide variation in the magnitude of HSPC mobilization by different inbred strains of mice in response to G-CSF. Following treatment of mice with 200 μg/kg/d G-CSF for 5 days, Roberts *et al.* (1997) observed a 10-fold range in the number of circulating progenitor cells between different inbred strains of mice, with the mobilization efficacy roughly aligning in the following order: DBA > 129Sv > BALB/c = SJL > C57Bl/6 = C3H/He. Similarly, Broxmeyer and colleagues (2005) reported that the combination of G-CSF and plerixafor induced significantly greater mobilization of HSPCs in DBA mice compared with either C57Bl/6 or C3H/He. Although the exact mechanism/s for this large interstrain variation remains unresolved, both genetic determinants and the size of the stem cell pool play a role in the efficiency of mobilization by G-CSF (reviewed in Herbert *et al.*, 2008). Because of the broad variability in mobilization efficiency by different strains of mice, it is preferable to test at least two strains of mice that differ in responsiveness to G-CSF when setting up mobilization experiments (Herbert *et al.*, 2008).

3.2. Selection of humans for HSPC mobilization

At our institution, eligible donors are between the ages of 18 and 70 years inclusive with evidence of adequate organ function (left ventricular ejection fraction more than 40%, formal pulmonary function testing showing a forced expiratory volume in 1 s [FEV1], more than 50% of predicted and a diffusing lung capacity for carbon dioxide [DLCO], more than 40% of predicted [corrected for hemoglobin]), a serum creatinine clearance of more than 40% of normal, a total bilirubin less than two times normal or absence of hepatic fibrosis/cirrhosis, no evidence of a severe central or peripheral neurologic abnormality, no evidence of active infection, be HIV negative, and have an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1. Donors must give written consent in accordance with the Declaration of Helsinki on a study approved by the Human Studies

Committee at Washington University. In the case of studies involving plerixafor, the Food and Drug Administration (FDA) approved the study under an investigator-held investigational new drug application.

4. Flow Cytometric Enumeration of Mobilized HSPCs

Previous chapters in this journal (Hawley *et al.*, 2006; Lin and Goodell, 2006) and elsewhere (Ema *et al.*, 2006; Fukuda and Pelus, 2008; Herbert *et al.*, 2008; Robinson and van Os, 2008) provide current procedures used to phenotypically characterize and isolate candidate human and murine HSPCs. The reader is referred to these publications for detailed methodology. In the following, we first briefly summarize the most common phenotypes used to characterize murine and human HSPCs.

4.1. Flow cytometric enumeration of murine HSPCs

Among the subsets that define hematopoietic stem cells, CD34$^-$ c-kit$^+$ Sca-1$^+$ lineage marker$^-$ (CD34$^-$KSL) cells are regarded as one of the populations that have the highest enrichment of HSPCs in adult mouse BM (Giebel and Punzel, 2008; Weissman and Shizuru, 2008). More recently, Morrison and colleagues (Kiel *et al.*, 2005) have used markers from the SLAM family—CD150, CD244, and CD48—to differentiate stem cells from more committed progenitor cells. The most primitive murine stem cells were found to reside within the CD150$^+$CD244$^-$CD48$^-$ subpopulation.

4.2. Flow cytometric enumeration of human HSPCs

The enumeration of cells that express the cell surface marker CD34 present on human HSPCs is used to assess the adequacy of stem cell numbers for hematopoietic stem cell transplantation. In humans, the CD34$^+$ cell population contains progenitors committed to the myeloid, erythroid, megakaryoid, and lymphoid lineages, as well as primitive progenitors and stem cells capable of long-term reconstitution (Giebel and Punzel, 2008; Weissman and Shizuru, 2008). Although no adequate threshold exists, a minimum of 2.0×10^6 CD34$^+$ cells/kg body weight is used by many centers to ensure adequate neutrophil recovery after transplant (Gandhi *et al.*, 1999; Montgomery and Cottler-Fox, 2007; Tricot *et al.*, 1995). Additionally, 5×10^6 CD34$^+$ cells/kg has been considered by some to be the optimal target as it results in faster platelet recovery post transplant (Bensinger *et al.*, 1995; Brown *et al.*, 1997; Weaver *et al.*, 1995).

5. Dosing and Kinetics of HSPC Mobilization by G-CSF and Plerixafor

5.1. G-CSF

5.1.1. Mobilization of murine HSPCs by G-CSF

Mice are typically mobilized with recombinant human G-CSF (Amgen, Thousand Oaks, CA) diluted in phosphate buffered saline (PBS) with 0.1% low endotoxin bovine serum albumin (BSA, Sigma) and administered by daily subcutaneous injection for 5 days at a dose of 250 μg/kg (Molineux *et al.*, 1990). Although the mechanism by which G-CSF induces HSPC mobilization remains controversial, the absence of a mobilization response in CXCR4$^{-/-}$ BM chimeras indicates the absolute dependence of this chemokine receptor in G-CSF–induced HSPC mobilization (Christopher *et al.*, 2009).

Pegylated-G-CSF (pegfilgrastim, Neulasta, Amgen, Inc) is a longer-lasting variant of G-CSF and was approved by the FDA in the USA to prevent prolonged neutropenia following chemotherapy for nonhematological malignancies (Kroschinsky *et al.*, 2008). The 33-h plasma half-life of pegfilgrastim is substantially longer than the 4- to 6-h half-life of G-CSF due to decreased serum clearance (Zamboni, 2003). Peak mobilization of murine CFU-GM and CAFC by pegfilgrastim is observed 3 days after a single subcutaneous injection of 25 μg (de Haan *et al.*, 2000).

5.1.2. Mobilization of human HSPCs by G-CSF

When G-CSF is used alone for human HSPC mobilization, the recommended dose is 10 μg/kg subcutaneous daily (either as a bolus or continuous infusion) beginning at least 4 days before the first apheresis session and continued until the last apheresis session (Gazitt *et al.*, 1999) (Neupogen [filgrastim]). Circulating CD34$^+$ stem cell levels usually peak on the 5th day of G-CSF (Lane *et al.*, 1995). Administration of G-CSF at a dose of at least 10 μg/kg/day for 5 days is usually required to achieve the mobilization goal of 5 $\times$ 10^6 CD34$^+$ cells/kg of recipient body weight, a dose considered suitable for reproducible, rapid, and consistent engraftment of both neutrophils and platelets (Henon *et al.*, 1992; Schmitz *et al.*, 1996). Although the Food and Drug Administration (FDA) approved pegfilgrastim for the prevention of prolonged neutropenia after chemotherapy for nonhematological malignancies, its potential as a mobilizing agent is still being explored. In healthy donors, a single dose of 12 mg pegfilgrastim has been shown to mobilize CD34$^+$ stem cells with a similar magnitude and kinetics as standard G-CSF (Hill *et al.*, 2006; Kroschinsky *et al.*, 2005).

High-dose GCSF was investigated as a primary mobilization regimen throughout the 1990s (Kobbe *et al.*, 1999; Sheridan *et al.*, 1994; Zeller *et al.*, 1996). Although seldom used today for primary mobilization, high-dose GCSF regimens are occasionally employed for remobilization (Boeve *et al.*, 2004; Wang *et al.*, 2007). Doses ranging from 16 to 32 μg/kg subcutaneous daily to 12 to 16 μg/kg subcutaneous twice daily have been considered as high-dose regimes (Bensinger *et al.*, 2009).

5.2. Plerixafor

5.2.1. Mobilization of murine HSPCs by plerixafor

Plerixafor (Genzyme, Cambridge, MA) is supplied as a sterile isotonic aqueous solution at 10 mg/ml. Broxmeyer and colleagues (2005) showed that a single-dose administration of 5 mg/kg subcutaneous plerixafor induces rapid mobilization of hematopoietic progenitor cells (HPCs) and long term repopulating cells to the blood of mice, with maximal mobilization of the HSPCs occurring 1 h postinjection. In agreement with these published results, we found that treatment of 129 × B6 F1 mice with subcutaneous plerixafor results in rapid mobilization of white blood cells (WBC) and HPCs, with peak CFU-GM levels achieved 3 h after a single injection of 5 mg/kg plerixafor (Ramirez *et al.*, 2008). Furthermore, we reported that repetitive subcutaneous injection of 5 mg/kg plerixafor to mice every 24 h results in a similar mobilization of progenitors (CFU-GM) after each injection (Nervi *et al.*, 2009). Similar data were generated by Hubel *et al.* (2004) using normal human volunteers. These data demonstrate that subcutaneous plerixafor can be given daily resulting in similar kinetics and magnitude of progenitor mobilization with no obvious tachyphylaxis.

In a separate series of experiments, we tested the efficacy of murine HSPC mobilization following intravenous administration of 1, 3, or 5 mg/kg plerixafor (Ramirez *et al.*, 2008). Analysis of the dose–response relationship indicated that intravenous plerixafor resulted in more rapid mobilization (peak 1 h) than subcutaneous administration. Doses higher than 3 mg/kg intravenous plerixafor were lethal to the mice.

5.2.2. Mobilization of human HSPCs by plerixafor

The FDA approved plerixafor for use in combination with G-CSF to mobilize HSPCs in patients with non-Hodgkin's lymphoma and multiple myeloma undergoing autologous transplantation in December 2008. Subcutaneous injection of 240 μg/kg plerixafor is initiated after the patient has received 10 μg/kg/day G-CSF for 4 days, followed by leukapheresis beginning 11 h after drug treatment (MOZOBIL [plerixafor injection]).

We recently published results from a Phase II study evaluating the safety and efficacy of plerixafor for CD34^{+} stem cell mobilization in allogeneic transplantation (Devine *et al.*, 2008). Twenty-five donors were treated with a single subcutaneous dose of 240 μg/kg plerixafor and underwent apheresis 4 h later, with collection of enough stem cells for transplant (defined as $>2 \times 10^{6}$ CD34^{+} cells/kg) in two-thirds of the donors. Twenty patients with hematologic malignancies received plerixafor-mobilized stem cell products with no adverse events. Although the CD34^{+} doses obtained were lower than that observed with a standard G-CSF mobilization regimen, the plerixafor-mobilized allografts functioned well and promoted rapid and durable multilineage hematopoiesis in the recipients.

In our Phase II study discussed above, only 16 of the first 24 donors mobilized with subcutaneous plerixafor (240 μg/kg) collected the minimal required target of 2×10^{6} CD34^{+} cells/kg in a single apheresis (Devine *et al.*, 2008). Based on preliminary data suggesting higher ($\sim$twofold) and earlier (1 h vs. 3 h) progenitor mobilization in mice after intravenous versus subcutaneous dosing of plerixafor (Ramirez *et al.*, 2008), we amended our trial and began testing the safety and efficacy of increasing doses of intravenous plerixafor (80, 160, 240, 320, 400, and 480 μg/kg over 30 min) on the kinetics and magnitude of allogeneic HSPC mobilization. In an ongoing Phase I safety evaluation of intravenous plerixafor, allogeneic related donors are initially mobilized with increasing doses of intravenous plerixafor. After 4 days of drug clearance, the same donors are then mobilized with a single subcutaneous dose of 240 μg/kg plerixafor, and collected cells are used as a source of stem cells for transplantation. Consistent with our hypothesis, patients treated intravenously with 240 μg/kg plerixafor had higher peak levels of CD34^{+} cells/μl blood at every time point evaluated compared to the same plerixafor dose administered subcutaneously (Rettig *et al.*, 2008). Furthermore, we have noted a clear dose–response effect of increasing doses of intravenous plerixafor. Of the seven donors who received 320 μg/kg intravenous plerixafor, all achieved peak levels of CD34/kg greater than 20 CD34/μl (range 22 to 38/μl), a level that we as well as others have shown is highly correlated with achieving more than 2×10^{6} CD34/kg after a single apheresis (Bensinger *et al.*, 2009; Pusic *et al.*, 2008). Since no related dose-limiting toxicity has yet been determined, we plan to complete the final two intravenous dose cohorts (400 μg/kg and 480 μg/kg). These encouraging studies suggest that intravenous plerixafor may be a more effective mobilizing agent with a low side effect profile. We predict, based on this preliminary data, that the optimal dose of intravenous plerixafor will result in similar rates of achieving 2×10^{6} CD34/kg after a single apheresis procedure compared to G-CSF in less time (4 h vs. 5 days).

6. Flow Cytometric Analysis of CXCR4 Expression on Human CD34$^+$ Subsets

A variety of cell types express the CXCR4 receptor, including peripheral blood lymphocytes (B cells and T cells), monocytes, neutrophils, pre–B cells, mast cells, CD34$^+$ HPCs, endothelial cells, intestinal and alveolar epithelial cells, astrocytes, microglia, and neurons (Khan *et al.*, 2007). CXCR4 receptors cycle continuously to and from the cell surface in a ligand-independent manner, with the majority of CXCR4 being stored in an intracellular pool (Busillo and Benovic, 2007; Marchese *et al.*, 2008; Zhang *et al.*, 2004). The function of these large stores of intracellular CXCR4 remains unclear.

In our recently published Phase II study evaluating the safety and efficacy of plerixafor for CD34$^+$ stem cell mobilization in allogeneic transplantation (Devine *et al.*, 2008), eight normal donors were mobilized sequentially with plerixafor and G-CSF. These donors initially received one subcutaneous injection of 240 μg/kg plerixafor, followed by leukapheresis beginning 4 h after drug treatment. After 10 days of drug clearance, the same donors were mobilized with 5 days subcutaneous injection of 10 μg/kg/day G-CSF, and leukapheresed on day 5. Interestingly, we found via flow cytometry that plerixafor mobilized a unique population of CD34^{dim} cells which were present in ~3- to 10-fold higher numbers compared to G-CSF mobilized CD34$^+$ cells (Rettig *et al.*, 2008). We further characterized CD34 immunoselected cells obtained after plerixafor or G-CSF mobilization of normal human donors by staining for CD34-APC and CD45RA-FITC. This staining and gating approach has allowed us to separate CD34$^+$ cells in plerixafor mobilized products into three separate subsets (only two in G-CSF mobilized grafts), with the CD34^{dim}CD45RA$^+$ subset relatively specific to plerixafor compared to G-CSF mobilized products (Fig. 3.1). Of interest, two of the key molecules responsible for stem cell homing, retention, and trafficking, CXCR4 and VLA-4, were significantly overexpressed in the CD34^{dim}CD45RA$^+$ subset compared to the CD34$^+$CD45RA$^-$ and CD34$^+$CD45RA$^+$ cells (Fig. 3.1). Others have shown that CD34$^+$CD45RA$^+$ cells represent more committed progenitors (reviewed in Blom and Spits, 2006; Weissman and Shizuru, 2008), with two different CD34^{dim}CD45RA$^+$ progenitor cell subsets having been described in the literature (Blom *et al.*, 2000; Freud *et al.*, 2005). Ongoing studies in the lab are further characterizing the CD34^{dim}CD45RA$^+$ progenitor cell subset preferentially mobilized by plerixafor. Below we describe in detail our method to purify and phenotype these different CD34$^+$ stem cell subsets.

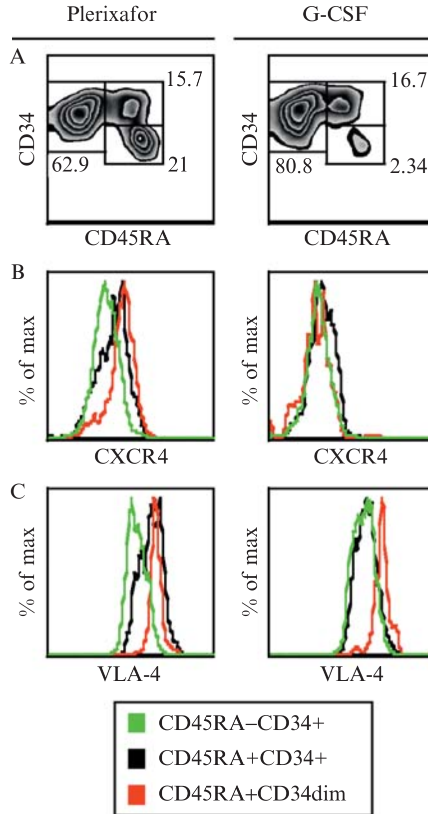

Figure 3.1 Coexpression of CD45RA on human $CD34^+$ cells identifies the $CD34^{dim}$ subset. (A) Healthy donors were treated with a single injection of 240 μg/kg AMD3100 or given 10 μg/kg/day G-CSF for 5 days. $CD34^+$ cells from leukapheresis products were purified by CD34 immunoselection using an autoMACS device and the expression of CD34 and CD45RA was evaluated by flow cytometry. $CD45RA^+CD34^{dim}$ cells are enriched in AMD3100-mobilized products. (B-C) $CD45RA^+CD34^{dim}$ cells from AMD3100-mobilized products express high levels of surface CXCR4 (B) and VLA-4 (C).

6.1. Evaluation of cell surface CXCR4 on human $CD34^+$ cell subsets

Aliquots of leukapheresis products are obtained in evacuated tubes coated with ethylene-diaminetetra-acetic acid (EDTA) or sodium heparin after informed consent in conformity with a human subjects protocol approved by an institutional review board. Rapid processing of samples is particularly important, since surface expression of CXCR4 may increase with time due to release from intracellular stores (Forster *et al.*, 1998; Shalekoff and Tiemessen, 2001). Cold (4°) storage stabilizes human $CD34^+$ cells.

1. Pass leukapheresis product through 30 μm nylon mesh (Miltenyi Pre-Separation Filters, #130-041-407) into a sterile 50 ml conical tube to remove cell clumps. Dilute cells by adding 20 to 30 ml of cold running buffer (phosphate-buffered saline supplemented with 0.5% bovine serum albumin and 2 m*M* EDTA, stored at 4°).
2. Perform a viable cell count on a hemacytometer.
3. Pellet cells at $300\times g$ for 5 min at 4°.
4. To prepare the cells for magnetic selection, decant supernatant and resuspend the cell pellet in a final volume of 300 μl of running buffer per 10^8 cells.
5. Isolate $CD34^+$ cells by positive selection using a CD34 Microbead Kit (cat. no. 130-046-702, Miltenyi Biotec, Auburn, CA) and autoMACS Separator (Miltenyi Biotec) according to the manufacturer's instructions. Set aside an aliquot of $\sim 10^6$ cells immediately before application to the autoMACS separator to use a pre-sort control for flow cytometry. Run sample through the autoMACS separator using the "posseld" (double-positive selection) program and collect both the positive (enriched $CD34^+$ cells) and negative ($CD34^-$ cells) fractions.
6. Perform a viable cell count on both the positive and negative fractions using a hemacytometer. After CD34 positive selection of ~6 ml of leukapheresis material, we typically obtain $\sim 5 \times 10^6$ and $\sim 2 \times 10^6$ $CD34^+$ cells from G-CSF (10 μg/kg/day × 5 days) and plerixafor (240 μg/kg subcutaneous) mobilized donors, respectively.
7. Label tubes and aliquot cells for flow cytometry as described in Table 3.2. Approximately 10×10^5 $CD34^-$ cells (negative sort) are used per tube to setup the instrument (compensation controls). In contrast, because of the limited number of $CD34^+$ cells obtained, we usually only aliquot ~0.5 to 1×10^5 $CD34^+$ cells (positive sort) per tube for the gating controls (fluorescence minus one controls) and experimental sample. All samples are placed in a final volume of ~100 μl of running buffer for flow cytometry analysis. Negative gating controls are analyzed to establish the level of background fluorescence resulting from autofluorescence and nonspecific antibody binding. Furthermore, fluorescence minus one gating controls are preferred over isotype controls because isotype controls are not always matched to the concentration of the test monoclonal antibody (mAb).

Extracellular staining of cells is performed as described in Table 3.2 by the addition of the following antihuman monoclonal antibodies (all obtained from BD Biosciences): CD4-FITC (clone RPA-T4, cat. no 555346), CD4-PE (clone RPA-T4, cat. no. 555347), CD4-APC (clone RPA-T4, cat. no. 555349), CD45RA-FITC (clone HI100, cat. no. 555488), CXCR4-PE (clone 1D9, cat. no. 551510), and CD34-APC (clone 581, cat. no. 555824). The amount of antibody added to each sample

Table 3.2 Staining setup for evaluation of CXCR4 on $CD34^+$ cell subsets

Tube no.	Sample	No. cells ($\times 10^5$)	FITC	PE	Viability	APC
Compensation controls						
1	$CD34^-$	10	—	—	7-AAD	—
2	$CD34^-$	10	CD4	—	7-AAD	—
3	$CD34^-$	10	—	CD4	7-AAD	—
4	$CD34^-$	10	—	—	7-AAD	CD4
Gating controls						
5	$CD34^+$	0.5	—	CXCR4	7-AAD	CD34
6	$CD34^+$	0.5	CD45RA	—	7-AAD	CD34
7	$CD34^+$	0.5	CD45RA	CXCR4	7-AAD	—
Experimental samples						
8	pre	10	CD45RA	CXCR4	7-AAD	CD34
9	$CD34^-$	0.5	CD45RA	CXCR4	7-AAD	CD34
10	$CD34^+$	10	CD45RA	CXCR4	7-AAD	CD34

7-AAD, 7-amino-actinomycin D; APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

is adjusted according to the number of cells used per the manufacturer's instructions. Since $CD34^+$ cells are immunoselected using the antihuman CD34 clone QBEND/10, post-sort analyses of CD34 expression must be performed using a separate mAb clone. The APC-conjugated anti-CD34 clone 581 provides a very bright signal that can be easily distinguished from the negative gating control. Additionally, the mAb most commonly used to study cell surface CXCR4 expression, clone 12G5, does not bind in the presence of plerixafor (Khan *et al.*, 2007). Clone 12G5 binds to an epitope on the second extracellular loop of CXCR4 that overlaps the plerixafor binding site on the extracellular loop 2 and the adjacent transmembrane segment TM4. Therefore, we use a separate clone, 1D9, which is not inhibited by plerixafor. The epitope recognized by antibody 1D9 is contained within the N-terminus of CXCR4 (Forster *et al.*, 1998).

1. Incubate samples for 30 min at 4° in the dark.
2. Remove unreacted antibodies by washing the cells twice in 3 ml of running buffer. After the final wash, decant the supernatant and resuspend the cells in ~300 μl of running buffer. Keep samples on ice until analysis.
3. We assess cell viability concomitantly with flow cytometry evaluation of stained cells by the addition of 7-amino-actinomycin D (cat. no. 559925, BD Biosciences).
4. Analyze the samples on a flow cytometer equipped for excitation wavelengths of 488 and 633 nm.

7. Functional Characterization of Mobilized HSPCs

7.1. *In vitro* assays of differentiation

In vitro assays of differentiation have been developed to quantify murine and human HSPC content (Sutherland *et al.*, 1989). In short-term colony-forming cell (CFC) assays, test samples are cultured in a semisolid matrix supplemented with nutrients and cytokines for ~2 weeks at 37°. During this culture period, CFCs proliferate and produce discrete cell clusters or colonies of morphologically recognizable daughter cells that can be quantified by light microscopy. Based on the selection of the appropriate media and culture conditions, CFC assays can be used to quantify myeloid multipotential progenitors (CFU-GEMM and CFU-GM) and lineage-restricted progenitors of the erythrocyte (CFU-E and BFU-E), granulocyte (CFU-G), monocyte-macrophage (CFU-M), megakayocyte (CFU-Mk), and B-cell (CFU–pre-B) lineages.

The standardized short-term colony assays discussed above easily quantify lineage-committed progenitors, but are not adequate for the detection of more primitive HSPCs. Two assays, the cobblestone-area–forming-cell (CAFC) assay (de Haan *et al.*, 2002) and the long-term culture-initiating cell (LTC-IC) assay (Lemieux *et al.*, 1995; Sutherland *et al.*, 1991), have been developed to measure more primitive stem cell frequencies. Both the CAFC and LTC-IC assays rely on adherent stromal cells for hematopoietic support and are quantified *in vitro* based on their capacity to generate myeloid cells for at least 5 weeks of culture. Additionally, the LTC-IC assay can be used in a quantitative manner by limiting dilution analysis to provide an estimate of the primitive cell pool within a product (Coulombel, 2004). The reader is referred to previous chapters in this series (Broxmeyer *et al.*, 2006) and elsewhere (Miller *et al.*, 2008; van Os *et al.*, 2008) for detailed descriptions of the CFC, CAFC, and LTC-IC procedures (see also www.stemcell.com/technical/manuals.asp).

7.2. Transmigration assays

Trafficking of HSPCs to the BM following transplantation is believed to be a critical step for hematopoietic reconstitution. Studies by Voermans *et al.* (2001) showed that enhanced *in vitro* migration of human CD34$^+$ cells to CXCL12 was associated with improved *in vivo* hematopoietic recovery. Since CXCL12-induced migration is not dependent on CXCR4 expression levels alone (Voermans *et al.*, 2001), and treatment with plerixafor, G-CSF or other mobilizing agents that target the CXCL12/CXCR4 interaction alter CXCL12 signaling, others and we often test the ability of

mobilized HSPCs to migrate to CXCL12 using transwell migration assays. We perform these assays according to the protocol described elsewhere by Fukuda and Pelus (2008).

7.3. Transplantation assays for mouse and human HSPCs

Long-term repopulating stem cells are defined by their ability to self-renew and to differentiate into mature cells of all hematopoietic lineages. The definitive assay for stem cell activity in a test sample is the complete and sustained (>6 months) reconstitution of all hematopoietic lineages in irradiated recipients by transplanted HSPCs (Herbert *et al.*, 2008; Purton and Scadden, 2007). The most common type of transplantation assay used to measure murine primitive stem cell activity is the competitive repopulation assay (Harrison, 1980). This assay measures the functional potential of an unknown "test" source of HSPCs (e.g., mobilized grafts) against a set known number of whole BM cells. The competing cells ensure the survival of lethally irradiated recipients transplanted with a low number of test HSPCs and allow quantification of the reconstitution activity. For donor versus host identification in transplantation assays, investigators commonly use C57BL/6 (B6) mice congenic for the CD45 (Ly5, common leukocyte antigen) locus to discriminate among the three potential sources of stem cells (test cells, competitor BM cells, and the host). We routinely use C57BL/6 ($CD45.2^{+}$) as recipients, congenic C57BL/6 ($CD45.1^{+}$) mice for mobilization, and hybrid C57BL/6 ($CD45.1^{+}CD45.2^{+}$) mice as BM donors. The number of repopulating units (RU) in the test sample is then determined by measuring the contribution of the test sample to donor chimerism at various time points after transplantation (Harrison *et al.*, 1993; Purton and Scadden, 2007; Yuan *et al.*, 2005). The value of the repopulating unit is indicative of the amount of repopulating activity within the test sample.

Although determination of the repopulating unit provides important information about the overall function of a test sample, it does not provide information on the quantity of primitive stem cells within the graft. The frequency of stem cells in an unknown test sample can be determined by performing limiting dilution competitive repopulation assays. In these studies, a series of dilutions of the test source are again competed against a set number of competing BM cells. The number of mice negative for reconstitution in each test cell dose is determined, and the frequency of HSPCs (competitive repopulating units, CRU) is estimated using Poisson statistics (Purton and Scadden, 2007; Szilvassy *et al.*, 1989, 1990; Taswell, 1981).

The most definitive test of long-term hematopoietic stem cells potential is the serial transplantation assay (Purton and Scadden, 2007). In this assay, the test sample is transplanted into sequential serial transplant recipients, and

the ability of the transplanted population to sustain hematopoiesis is determined.

Since the limiting dilution competitive repopulation assay can be used to incorporate all three types of the long-term repopulating stem cell assays discussed above (RU, CRU, and serial transplantation), we will describe the assay in greater detail in the following.

7.3.1. Limiting dilution competitive repopulation assay

Wildtype C57BL/6J (CD45.2$^+$) and a congenic strain of C567BL/6 that have the CD45.1 gene (B6.SJL-PtPrc*Pep3BoyJ) are obtained from The Jackson Laboratory (Bar Harbor, ME). Hybrid C57BL/6J $\times$ B6.SJL-PtPrc*Pep3BoyJ F1 (CD45.1$^+$/CD45.2$^+$ heterozygous) are bred at our animal facility. The Ly5/CD45 antigen is expressed on all hematopoietic cells except erythrocytes, and polymorphism between CD45.1 and CD45.2 provides a quick and convenient method for detecting donor cells within leukocytes of recipients using flow cytometric techniques. All mice are 8 to 10 weeks old and sex-matched.

Wildtype C57BL/6J (CD45.2$^+$) recipients are exposed to a lethal dose of total body irradiation from 12 to 24 h before transplantation. Since irradiation toxicity levels can be variable between institutions, it is preferable that all investigators assess the level of radiation that their mice can tolerate without any morbidity and mortality. Typical irradiation doses range for C57BL/6 mice range from 1000 cGy to 1100 cGy TBI. At least 16 to 20 mice are irradiated per experiment.

Low-density mononuclear cells (LDMNCs) are isolated from mobilized B6.SJL-PtPrc*Pep3BoyJ (CD45.1$^+$) mice using murine lympholyte (Cedarlane Laboratories, Burlington, Ontario, Canada). Approximately 2×10^7 total LDMNCs are needed to inject at least four mice at a minimum of three different cell doses. For plerixafor, we typically treat 20 to 25 CD45.1$^+$ B6 donor mice with 5 mg/kg subcutaneous plerixafor and harvest peripheral blood 3 h later. For GCSF, we treat 10 to 15 CD45.1$^+$ B6 donor mice with G-CSF (250 μg/kg/day) for 5 days and harvest peripheral blood 4 h after the last injection of G-CSF.

Isolate BM cells aseptically from C57BL/6J $\times$ B6.SJL-PtPrc*Pep3BoyJ F1 (CD45.1$^+$/CD45.2$^+$) mice. We sacrifice 1 CD45.1$^+$/CD45.2$^+$ B6 BM donor mouse for every 15 to 20 lethally irradiated CD45.2$^+$ B6 mice undergoing transplantation.

LDMNCs from mobilized CD45.1$^+$ B6 mice are mixed with unfractionated CD45.1$^+$/CD45.2$^+$ B6 competitor BM cells. At least four mice at a minimum of three different cell doses should be evaluated to allow statistical comparison of test (CD45.1$^+$) cell engraftment between treatment groups at limiting dilution (Purton and Scadden, 2007). Most investigators inject between 2 to 5×10^5 competing BM cells per mouse. However, the number of LDMNCs injected per mouse is variable between institutions

and each mobilizing agent. For plerixafor and G-CSF mobilized grafts, we and others (Broxmeyer *et al.*, 2005) have set the ratio of donor ($CD45.1^{+}$) blood cells to competitor ($CD45.1^{+}/CD45.2^{+}$) BM cells as the number or LDMNCs in three, two, or one donor mice to a constant number of 5×10^{5} competitor BM cells ($CD45.1^{+}/CD45.2^{+}$). For example, at a 3:1 ratio, we mix the number of LDMNCs obtained from the peripheral blood of three donor mice ($CD45.1^{+}$) with 5×10^{5} competitor BM cells ($CD45.1^{+}/CD45.2^{+}$). Others mix 5×10^{5} competitor BM cells with 2×10^{6}, 1.5×10^{6}, or 1×10^{6} LDMNCs to yield LDMNC to BM ratios of 4:1, 3:1, or 2:1, respectively (Fukuda *et al.*, 2007). Pilot experiments are recommended to obtain the range of LDMNCs required to achieve durable test sample engraftment. Four B6 ($CD45.2^{+}$) mice that received 1000 cGy TBI and 5×10^{5} competitor BM cells ($CD45.1^{+}/CD45.2^{+}$) without donor test cells were used as controls.

Hematopoietic repopulation is evaluated monthly for at least 6 months to demonstrate long-term multilineage reconstitution in the $CD45.1^{+}$ and $CD45.1^{+}/CD45.2^{+}$ donor cell subsets. Multilineage analysis is performed on the blood by flow cytometry using antimouse monoclonal antibodies against CD45.1, CD45.2, and the lineage markers B220 (B lymphoid), CD3 (T lymphoid), Mac1 (monocyte/macrophage), and Gr1 (granulocyte). At least 20,000 events are acquired on a flow cytometer. Since myeloid progenitors and their progeny have short half-lives compared to lymphoid progeny, it is important to demonstrate myeloid reconstitution in the test-cell subset following transplantation. Furthermore, Bryder *et al.* (2004) demonstrated that the RB6-8C5 mAb detecting Gr-1 also binds to a subpopulation of $CD3^{+}CD8^{+}$ T cells present in the peripheral blood. Therefore, granulocyte reconstitution should be defined as Gr-1^{+} cells negative for expression of T-cell markers like CD3.

The percentage of chimerism is calculated based on flow cytometry data as follows: % chimerism = (% test donor cells) × 100 / (% test donor cells + % competitor cells). Most investigators consider that primitive stem cells are present in the test donor cells when the percent chimerism is greater than 1% for all myeloid (granulocytes and macrophages), B-lymphoid, and T-lymphoid lineages at 6 months after transplantation.

The number of repopulating units (RU) in test donor cells is calculated according to the method of Harrison *et al.* (1993) as follows: % chimerism = (% chimerism) × (no. of competitor cells/10^{5}) /(100 – % chimerism). One RU is defined as the amount of repopulating activity in 10^{5} BM cells from wildtype mice (Ema *et al.*, 2006; Purton and Scadden, 2007).

In limiting dilution assays, the frequency of competitive repopulating units (CRU) among test donor cells is estimated on the basis of Poisson statistics; the ranges of CRUs are given as 95% confidence intervals. Stem-Cell Technologies has developed a program, L-Calc, to aid in the data analysis of limiting dilution assays. This software is free to download from

the company website. Of note, CRU and RU are different (Ema *et al.*, 2006; Purton and Scadden, 2007). CRU measures the quantity of HSPCs, whereas RU measures the functional quality of HSPCs. Furthermore, since short-term repopulating cells can reconstitute multiple lineages for at least 16 weeks, most researchers determine RU and CRU values from data collected at least 6 months post-transplantation.

It should be noted, that noncompetitive primary transplantation assays can be performed to mimic how transplants are performed clinically. In a noncompetitive transplant, test cells (typically 1 to 2×10^6) are injected into lethally irradiated congenic recipients in the absence of competing BM cells. Although the primary endpoint in noncompetitive transplants is the time to recovery of peripheral blood neutrophils, platelets, and hemoglobin, donor chimerism can also be determined as described.

7.3.2. Secondary transplantation

Wildtype C57BL/6J (CD45.2$^+$) recipients are exposed to a single dose of lethal (1000 cGy) total body irradiation from a ^{37}Cesium source at a rate of 95 cGy/minute 12 to 24 h before transplantation.

Primary recipient mice are sacrificed at 6 months post-transplant and the contents of their femurs are pooled within the respective treatment groups.

Pooled BM cells (1×10^6) are injected via the tail vein using a 27-gauge needle in 0.2 ml of PBS within 12 h after irradiation of C57BL/6J (CD45.2$^+$) recipients. If possible, at least 10 secondary recipients should be injected with BM cells harvested from the primary recipients.

The proportions of CD45.2 donor and CD45.1 competitor cell engraftment in the secondary recipients were measured at 2, 6, 14, and 27 weeks using the same methods.

Tertiary transplantations were carried out in the same manner.

7.3.3. Immune-deficient mouse models to study human stem cell–repopulation capacity

Several xenotransplantation models have been developed as surrogate assays of human HSPC activity, with the majority relying on the use of different strains of immunodeficient mice with various degrees of residual innate immunity. Nonobese diabetic (NOD) mice crossed with severe combined immunodeficient (SCID) mice represent the most accepted and widely used immune-deficient animal for quantitative comparison of human HSPC activity (Cashman *et al.*, 1997; Larochelle *et al.*, 1996; Pflumio *et al.*, 1996). NOD/SCID mice stringently engraft only primitive human hematopoietic stem cells (scid-reconstituting cells [SRC]) that repopulate the BM with predominantly CD34$^+$CD19$^+$ pro-B cells exhibiting a poor capacity to terminally differentiate, and to a lesser degree, myeloid cells. In contrast to the SRC, more committed human progenitor populations are able to engraft NOD/SCID mice back-crossed with the β_2-microglobulin–null

(β_2m^{null}) allele (Kollet *et al.*, 2000, 2001). These NOD/SCIDβm^{null} mice exhibit a more absolute immunodeficiency than NOD/SCID mice and have virtually no NK cell function. In fact, compared to NOD/SCID controls, NOD/SCIDβ_2m^{null} mice support a greater than 10-fold higher level of SRC frequency upon transplantation of small numbers (8×10^4 cells) of human cord–blood mononuclear cells and become reconstituted with lymphoid CD45$^+$CD19$^+$ cells (no T cells) and myeloid CD45$^+$CD33$^+$ cells. This enhanced SRC frequency in NOD/SCIDβm^{null} mice is caused by the increased engraftment of human myeloid and lymphoid short term repopulating hematopoietic cells (Eaves *et al.*, 2001; Glimm *et al.*, 2001).

Two major limitations of the NOD/SCIDβm^{null} xenograft model are their poor reproduction rate and short life span (approximately 6 months due to accelerated thymic lymphomagenesis). One alternative to using NOD/SCIDβm^{null} mice for measuring human HSPC activity is to treat NOD/SCID mice with a monoclonal antibody (mAb) against the interleukin-2 receptor βchain (IL-2Rβ, CD122). The anti-CD122 mAb eradicates CD122-expressing cell populations, including NK cells and macrophages that mediate a negative effect on human engraftment. Compared to NOD/SCIDβ_2m^{null} mice, anti-CD122 treated NOD/SCID mice exhibit a nearly threefold greater human cell engraftment upon transplantation of human cord–blood mononuclear cells (McKenzie *et al.*, 2005). A second alternative to using NOD/SCIDβ_2m^{null} mice in SRC assays is to use NOD/SCID mice harboring a complete null mutation of the interleukin 2 receptor common γ chain (NOD/SCID/γ_c^{null}) (Ito *et al.*, 2002; Shultz *et al.*, 2005). Similar to NOD/SCID-β2m^{null} mice, NOD/SCID/γ_c^{null} mice have reduced activities and numbers of NK cells. However, unlike NOD/SCID-β2m^{null} mice, NOD/SCID/γ_c^{null} mice can survive long term (15 months) because they do not develop thymic lymphomas, have a reproduction rate similar to normal wildtype mice, and exhibit multilineage engraftment of mature and functional CD3$^+$CD4$^+$ and CD$^+$CD8$^+$ T cells, Ig$^+$ B cells, NK cells, monocytes/macrophages, and plasmacytoid dendritic cells following transplantation of human CD34$^+$ HSCs. Furthermore, since NOD/SCID/γ_c^{null} mice require no anti-CD122/IL-2Rβ monoclonal antibody treatment for human cell engraftment, they provide a significant cost advantage over NOD/SCID mice.

NOD/LtSz-*Prkdc^{scid}/Prkdc^{scid}* (NOD/SCID), NOD/LtSz-*Prkdc^{scid}/Prkdc^{scid}βm^{null}* (NOD/SCID-β2m^{null}), and NOD.Cg-*Prkdc^{scid} Il2rg^{tm1Wjl}*/SzJ (NOD/SCID/γ_c^{null}) mice are obtained from Jackson Laboratories (Bar Harbor, ME), and bred at our animal facility. NOD/SCID mice are known to be ''leaky,'' and sometimes develop mouse CD3$^+$ T cells that can impede human cell engraftment. Therefore, we screen the peripheral blood of all NOD/SCID mice by flow cytometry with antimouse monoclonal antibodies (CD45, CD3, and DX5) and eliminate any mice exhibiting more than 1% CD3$^+$ T cells.

All immunodeficient mice are housed in a specific pathogen-free facility in sterile microisolator cages, and given autoclaved food and water *ad libitum*. All manipulations are performed aseptically on a laminar flow bench.

We condition 8- to 10-week-old NOD/SCID, NOD/SCIDβ_2m^{null}, and NOD/SCID/γ_c^{null} mice with 300 cGy, 300 cGy, and 250 cGy of single-dose total body γ irradiation (TBI), respectively, using a Shepard Mark IV Cesium137 irradiator. However, since irradiation toxicity levels can be variable between institutions, it is preferable that each investigator assesses the level of radiation that their mice can tolerate without any morbidity and mortality. Typical irradiation doses range from 250 cGy to 300 cGy TBI.

NOD/SCID mice treated with anti-CD122 antibody are given injections of 200 μg purified antibody into the intraperitoneal cavity immediately after irradiation. The anti-CD122 monoclonal antibody generated from the hybridoma cell line TM-β1 can be purchased from Bio Express Inc. (West Lebanon, NH).

Human mobilized peripheral blood mononuclear cells and purified CD34$^+$ cells are injected via the tail vein using a 27-gauge needle in 0.2 ml of PBS within 12 h after irradiation. A range of human MNC (10^6 to 40×10^6 cells) or purified CD34$^+$ cells (2×10^4 to 1×10^6 cells) are injected into quadruplicate mice at a minimum of three different doses per donor to allow direct statistical comparison of human cell engraftment between treatment groups at limiting dilution. Control mice are irradiated but do not receive human cells.

Ten to 12 weeks after transplantation, BM (femurs and tibias), spleen, and peripheral blood are recovered, single cell suspensions prepared, and numbers of total nucleated cells are determined using a hemacytometer.

The appropriate dilution of antibodies, as titered against human or mouse peripheral blood mononuclear cells, are incubated with 1 to 5×10^5 cells for 30 min at 4° and then washed two times in phosphate-buffered saline (PBS) plus 0.5% bovine serum albumin. At least 10,000 events are acquired on a flow cytometer. Antimouse CD45 and antihuman CD45 mAb are used to determine the number of mouse and human hematopoietic cells, respectively. Engrafted mouse BM is further analyzed for the frequency of human B-lymphoid cells (CD20-FITC, CD19-PE), myeloid cells (CD14-FITC, CD33-PE), T-lymphoid cells (CD4-FITC, CD8-PE), and primitive HSPCs (CD34-FITC, CD38-PE). All antibodies are purchased from Becton Dickinson (San Diego, CA). To accurately set up the cytometer, we mix equal numbers of BM cells from a control, untransplanted immunodeficient mouse with human PBMCs. These control samples are then stained individually with antihuman CD45 and antimurine CD45 or simultaneously with the antihuman CD45/antimurine CD45-lineage cocktails described.

The proportion of human cells in each mouse is calculated as follows: % huCD45$^+$ = [no. huCD45$^+$/(no. huCD45$^+$ + no. muCD45$^+$)]. Mice are considered engrafted when at least 1% of human CD45$^+$ cells are detected in the mouse BM. Short-term human reconstitution potential is measured by sacrificing and analyzing mice 6 weeks after transplantation.

Levels of human engraftment are reported as the mean ± SD for mice grouped according to transplanted cell numbers and compared using a Student's *t*-test. SCID repopulating cell (SRC) analysis is performed using the single-hit model and Poisson statistics at limiting dilution with 95% confidence intervals. Typically, data from three limiting dilution experiments are pooled and analyzed using L-Calc software (Stem Cell Technologies; free software download).

8. Concluding Remarks

Disruption of CXCL12/CXCR4 signaling is a critical step in HSPC mobilization by G-CSF, plerixafor, and additional agents in development. We (Devine *et al.*, 2008; Hess *et al.*, 2007; Rettig *et al.*, 2008) and others (Fruehauf *et al.*, 2006; Jin *et al.*, 2008; Pelus and Fukuda, 2008) have found intrinsic differences between HSPCs mobilized with plerixafor and G-CSF, including differences in cell surface markers, cell cycle, gene expression profiles, and NOD/SCID repopulating capacity. The optimum strategy for obtaining mobilized peripheral blood from donors is the subject of ongoing study.

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