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Insights into the biology of mobilized hematopoietic stem/progenitor cells (HSPC) through innovative treatment schedules of the CXCR4 antagonist AMD3100

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Abstract

Objective—The CXCR4 antagonist AMD3100 mobilizes hematopoietic stem/progenitor cells (HSPC) in several species. Few data are available on the biology of HSPC mobilized with AMD3100 as single agent. To further study the kinetics and properties of AMD3100-mobilized HSPC, and to explore the size of mobilizable pools of HSPC targeted by AMD3100, we studied the effect of a continuous infusion scheme with saturating doses of AMD3100 [AMDi].

Materials and Methods—Using established procedures, we evaluated mice mobilized with AMD3100, or those transplanted with AMD3100-mobilized HSPC.

Results—Relative to single-bolus AMD3100 [AMDb], the number of circulating CFU-C or CRU was dramatically higher after [AMDi]. During [AMDi], circulating CFU-C accumulated slowly, but after its discontinuation, CFU-C disappeared rapidly. Compared to BM-c-kit⁺ cells, AMD3100-mobilized (AMDb or AMDi) c-kit⁺ cells showed reduced expression of several cytoadhesion molecules, similar to G-CSF-mobilized c-kit⁺ cells. In contrast to the latter, expression of CXCR4 and CD26 were not reduced on AMD3100-mobilized c-kit⁺ cells. Bone marrow (BM) homing of [AMDi]-mobilized CFU-C was >50% increased over normal BM-CFU-C. Hematopoietic recovery after transplantation of [AMDi]-mobilized peripheral blood (MPB) was comparable to that of continuous infusion G-CSF-MPB. AMD3100-mobilized HSPC were predominantly in G₀, and partial BrdU labeling experiments documented under-representation of labeled cells (<5%) among [AMDb]-mobilized c-kit⁺ cells, suggesting that cycling cells in BM, or those that recently completed cell cycle, are not targeted for mobilization by AMD3100.

Conclusions—Our data demonstrate that [AMDi] is an efficacious mobilization scheme fully supporting transplantation demands and expand previous knowledge about properties and size of AMD3100-sensitive BM-HSPC pools.

INTRODUCTION

G-CSF mobilized HSPC are currently a widely used source of transplantable cells in the clinic. Donor preference and faster hematopoietic regeneration compared to bone marrow (BM) are

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among the reasons cited.[1,2] Although the mechanisms of G-CSF induced mobilization are complex and not fully defined, clearly an interference with CXCR4/CXCL12 mediated marrow retention pathways is mechanistically involved.[3,4] Inefficiency of G-CSF in a proportion of normal donors[5,6] or patients[7] as well as adverse effects of G-CSF, such as reactivation of autoimmune processes[8,9] and immune modulation of grafts leading to increased chronic GvHD in recipients,[10] have provided a rationale for targeting CXCR4/CXCL12 directly for the purposes of HSPC mobilization. A small-molecule receptor antagonist, AMD3100, was described, and HSPC mobilization by AMD3100 alone,[11-13] and especially in combination with G-CSF,[14,15] was demonstrated. Because of the relatively low efficiency of previous AMD3100 mobilization protocols (on average, 16 CD34+ cells/ μ l for single-dose AMD3100 vs. >100 CD34+ cells/ μ l for a 5-day course of once-daily Lenograstim in normal human donors),[13,16] properties of AMD3100 mobilized HSPC, or size and location of mobilizable pools, have not been thoroughly addressed, even though a study indicating the feasibility of apheresis of AMD3100 bolus mobilized allogeneic volunteer donors and of transplantation of such HSPC were recently published.[13] The purpose of the present studies was, therefore, to optimize AMD3100-based HSPC mobilization protocols and to explore certain transplantation-related properties (stem cell frequency, homing efficiency, engraftment kinetics) of AMD3100 mobilized HSPC in mice. Moreover, because of the rapid kinetics of mobilization by AMD3100, questions about AMD3100 sensitive pools, and the cycling status of HSPC in BM targeted for mobilization were pursued.

MATERIALS AND METHODS

Mice

B6x129 or C57Bl/6 wild-type mice were used for most experiments. In addition, we used C57Bl/6 (CD45.2) and B6.SJL (CD45.1) mice (Jackson Laboratories, Bar Harbor, ME) for competitive engraftment experiments. G-CSFR^{-/-} mice (a generous gift from D. Link, Washington University, St. Louis) and appropriate wild-type controls, were also used for some mobilization experiments; these mice were previously described.[17] Some mice were splenectomized under aseptic conditions with general anesthesia and postoperative analgesia. These mice were given ≥ 4 weeks to recover from surgery before experimentation. All animals were housed at the University of Washington Comparative Medicine Specific Pathogen-Free vivarium or at the Johann-Wolfgang-Goethe University Medical School vivarium, with autoclaved chow and water *ad libitum*. All procedures were done in agreement with IACUC approved protocols.

Mobilization

AMD3100 (Sigma-Aldrich, St. Louis, MO) was suspended in PBS/BSA, and either injected i.p. or filled into continuous-release model 2001 osmotic minipumps (Alzet, Palo Alto, CA), releasing 1 mg AMD3100/day for up to 9 days. rhG-CSF (Neupogen, Amgen, Thousand Oaks, CA) was similarly filled into osmotic minipumps and released at a dose of 100 μ g/kg*day. Osmotic minipumps were sterilely implanted into a subcutaneous pouch at the back under general anesthesia, as described.[18] Some of the G-CSFR^{-/-} animals received a single intraperitoneal injection of 4 mg (ca. 200 mg/kg) Cyclophosphamide.

Cells

Blood was drawn from the retroorbital plexus or from the saphenous vein. BM cells were recovered from femurs, tibias or pelvic bones of painlessly killed animals.

Colony Assay

Colony assays were grown in duplicate in commercially available growth-factor supplemented methyl cellulose medium (Stem Cell Technologies, Vancouver, BC) as described.[19] CFU-C (BFU-E, CFU-GM and CFU-mixed) were enumerated after 7 days.

Homing assay

Recipient mice were conditioned by lethal irradiation, received i.v. transplants, and marrow homing 20 hours after transplantation was enumerated, as described.[20] Homing results are displayed as the fraction of the total injected CFU-C homed to one femur.

Engraftment analysis

Non-competitive engraftment was tested by transplantation of lethally irradiated mice with suspensions of low doses (100 μ L) of mobilized blood cells followed by analysis of complete blood counts every 2-4 days. For CBC, 20-30 μ L of blood were drawn from the saphenous vein and analyzed on the Hemavet 950SF+ (Drew Scientific, Dallas, TX).

Limiting dilution CRU assay

CRU assays were done as previously described.[21] Lethally irradiated CD45.2 hosts, 8-10 per group, received a mix of limiting quantities of CD45.1 mobilized blood cells (total WBC from 2-18 μ L of mobilized blood) and 250,000 CD45.2 BM competitor cells. Pilot experiments had established these to be limiting doses for AMD3100 mobilized blood engraftment at the 1% level. After 16 weeks, the relative contribution of CD45.1 to total CD45 was established by flow cytometric analysis. Animals with >1% donor contribution were considered positive for donor cell engraftment. CRU frequency was calculated using LCA software (Stem Cell Technologies).

Migration Assay

Transwell migration through 5 μ m pore-size transwells (Corning Costar) was assessed after 3 or 4 h in DMEM+10% FCS $\pm$ SDF-1 (Peprotech, Rocky Hill, NJ), using flow cytometry or colony assays, as was previously described.[20]

Cell cycle analysis

c-kit+ cells from mobilized blood or BM were isolated by flow sorting (FACSaria, Becton-Dickinson, San Jose, CA), then stained with Acridine Orange and analyzed by flow cytometry. Cells were excited at 488 nm. DNA was detected in channel 1, RNA in channel 3 of a standard set-up FACSCalibur (Becton-Dickinson) flow cytometer.[22]

BrdU labeling

BrdU (Sigma-Aldrich) was dissolved in PBS/BSA and injected i.p. into mice at a dose of 0.167 mg/g. In some instances, additional injections of the same dose were given 3 and 6 hours later, to achieve higher BrdU marking. One hour before sacrifice, animals received an injection of AMD3100 (100 μ g) in PBS/BSA or PBS/BSA alone (controls). Animals were lethally exsanguinated and one femur was removed as a source of BM cells. Red cells were lysed, followed by staining for c-kit (2B8-APC, Becton-Dickinson), and processing for anti-BrdU (anti-BrdU-PE) staining according to manufacturer's instructions (Becton-Dickinson). Cells from not BrdU pulsed animals were processed in the same way and served as anti-BrdU antibody staining controls. Samples were analyzed by flow cytometry, by gating on APC+ cells and analysis of PE-fluorescence of these cells.

Flow cytometry

Cells were labeled with directly fluorescence-conjugated specific or isotype control antibodies (all from Becton-Dickinson, San Jose, CA) according to standard protocols and analyzed by multi-parameter flow cytometry (FACSCalibur, Becton-Dickinson) and CellQuest software (Becton-Dickinson).

Statistics

Descriptive statistics and t-tests were calculated using Excel (Microsoft, Redmond, WA). A $p < 0.05$ was considered statistically significant.

RESULTS

Highly efficient HSPC mobilization by continuous AMD3100 infusion

HSPC mobilization after a single bolus of AMD3100 peaked one hour after i.p. injection, at approximately three- to five-fold over baseline (1,000 CFU-C/mL). Circulating CFU-C normalized rapidly thereafter (Fig. 1A, left). 50 μ g AMD3100/mouse were required to significantly increase circulating CFU-C, and 100 μ g/mouse were sufficient for peak mobilization (Fig. 1A, right); further dose increase did not result in additional mobilization. The relative distribution between myeloid colony types was similar at baseline and at the peak of AMD3100 mobilization, with ca. 10-20% BFU-E, 80-90% CFU-GM, and rare mixed CFU-C (not shown), i.e. AMD3100 did not show a preference for the mobilization of either of the myeloid lineage progenitor cell populations. More importantly, repeated injections of maximally effective doses of AMD3100 12 hours apart for a total of 12 doses resulted in mobilization one hour later of the same number of CFU-C as after the first injection, i.e. without apparent CFU-C accumulation or exhaustion of the AMD3100-sensitive mobilizable pool (Fig. 1B, left). Similarly, when doses were given only 4 hours apart, i.e. the second dose was injected at the time that circulating CFU-C numbers had just returned to baseline, mobilization of the expected magnitude for a single bolus of AMD3100 was observed (Fig. 1B, right).

We reasoned that the low mobilization efficiency of bolus injections of AMD3100 was primarily due to the short biological half-life of AMD3100 and therefore attempted mobilization by continuously infused AMD3100. Ten hours after the beginning of the infusion, CFU-C were 2-3-fold higher than after a single bolus (not shown), were half-maximal after 24 hours, and on days 4-8, >10,000 CFU-C/mL (up to 20,000 CFU-C in some experiments, Fig. 1D) were in circulation, which was 10- to 20-fold more than after a single bolus, or >50-fold more than at baseline (Fig. 1C). This number of CFU-C in the total blood volume of a mouse is equivalent to the CFU-C contents of one third of a femur, or ca. 2% of the total CFU-C contents of a mouse at steady-state.[23] After discontinuation of the infusion (pump removal), circulating HSPC disappeared from circulation with remarkably rapid kinetics: CFU-C dropped by >80% within 6 hours, and had returned to baseline values by 24 hours (Fig. 1C). Importantly, not only progenitor cell mobilization was enhanced after continuous infusion of AMD3100, but also mobilization of repopulating cells (CRU): Using limiting-dilution competitive transplantation assays, we could demonstrate that continuous AMD3100 infusion mobilized >3.5-fold greater numbers of CRU than an AMD3100 bolus (Fig. 1F).

The number of CFU-C mobilized by continuous AMD3100 infusion thus compares favorably to that by continuous infusion of G-CSF (Fig. 1D), and far exceeds that achieved with bi-daily injections of G-CSF for 5 days.[24] Similarly to what was shown for sub-optimal dosing schedules of G-CSF,[24] synergistic mobilization was also observed for the combination of continuous G-CSF infusion for 5 days followed by a bolus of AMD3100 and analysis one hour later (Fig. 1D). As was pointed out, approximately 2% of CFU-C were in circulation during AMD3100 infusion. Thus the observation that the BM CFU-C content was not discernibly

different from baseline after 5 days of AMD3100 infusion (Fig 1D) was not unexpected. In contrast, however, despite equivalent mobilization by G-CSF alone, CFU-C content in BM of G-CSF infusion treated mice was reduced by approximately $\frac{1}{4}$, as previously described,[25] which is likely the result of additional effects of G-CSF on proliferation/differentiation and marrow remodeling. BM CFU-C content was more strongly reduced after the combination of G-CSF infusion plus AMD3100 bolus than after G-CSF alone (Fig. 1D), which likely reflects the potent mobilization (2 femur equivalents) with this combination of mobilizing agents.

Immunophenotype, in vitro migration and in vivo homing/engraftment of AMD3100-infusion mobilized HSPC

Expression of a number of surface markers on c-kit⁺ cells in steady-state BM, AMD3100-bolus mobilized blood, AMD3100-infusion mobilized blood or G-CSF-infusion mobilized blood were tested by flow cytometry. As previously described,[26] c-kit expression was reduced on G-CSF mobilized cells; in particular the prominent c-kit bright population in BM was not detected in blood. Similar observations were made for AMD3100 infusion and AMD3100 bolus mobilized blood. Whether this indicates that c-kit expression is diminished by mobilization, or that c-kit dim HSPC are preferentially mobilized was not clear. Cells were analyzed after gating on all c-kit positive cells. Overall, cytoadhesion molecules were relatively reduced on all mobilized c-kit⁺ cells, irrespective of mobilizing agent, to a degree that would likely affect functionality, although individual values varied between mobilization modalities (Table 1). This phenotype was previously described for G-CSF mobilized HSPC, and may suggest that a hypo-adhesive phenotype is characteristic of circulating HSPC, and possible a prerequisite for their egress from BM.[27] Of interest, the same characteristics are also found on “naturally” circulating HSPC. Also as previously reported for G-CSF mobilized HSPC and for HSPC circulating during steady-state,[28] AMD3100 mobilized cells expressed virtually no VCAM-1 (not shown), although the functional relevance of this remains unclear in the absence of a defined role for VCAM-1 on HSPC.

By contrast, CXCR4 mean fluorescence was slightly increased on AMD3100-bolus mobilized blood HSPC (but less so on AMD3100-infusion mobilized HSPC, Table 1), while it was dramatically down-regulated on G-CSF-mobilized HSPC. This finding was previously emphasized in monkeys,[29] with unclear significance as to its meaning for HSPC function. Therefore, in vitro responsiveness of HSPC to SDF-1 was tested functionally. As previously shown,[19] in vitro incubation of normal BM c-kit⁺ cells with AMD3100 almost completely blocked SDF-1 directed in-vitro transwell migration (Fig. 2A). In striking contrast, transwell migration of AMD3100 infusion-mobilized blood CFU-C towards SDF-1 was similarly efficient as that of BM-CFU-C (Fig. 2B), whereas superior migration of G-CSF mobilized HSPC was previously described, in spite of reduced surface expression of CXCR4.[20] As we have previously reported, CD26 was completely down-regulated on G-CSF mobilized HSPC, while CD26 on AMD-mobilized HSPC (by pump or by bolus) was similar to BM-HSPC, an observation which might contribute to the differential migratory phenotype of AMD3100 vs. G-CSF mobilized HSPC.

Factors guiding clinical outcomes of HSPC transplantation include stem cell frequency and in vivo performance of transplanted cells with respect to homing and repopulation, and these properties were therefore next addressed. Recovery of transplanted AMD3100 infusion-mobilized CFU-C in lethally irradiated hosts was tested 20 hours after transplantation. The efficiency of AMD3100 infusion-mobilized blood CFU-C homing was >50% greater than that of steady-state BM-derived CFU-C and of BM-cells from AMD3100 infusion-mobilized hosts (Fig. 3A), similar to observations for G-CSF infusion mobilized HSPC and for BM-HSPC from G-CSF treated donors (Fig. 3A). Engraftment kinetics of G-CSF or AMD3100 infusion-mobilized blood HSPC were also compared. Lethally irradiated hosts received transplants of

100 μ l mobilized blood per recipient (either AMD3100 infusion or G-CSF infusion mobilized), equivalent to approximately 1,000 or 1,400 CFU-C in recipients of AMD3100 infusion- or G-CSF infusion-mobilized blood, respectively, a number which was appropriately estimated to ensure survival of all hosts. Leukocyte counts and hematocrit recovered equally rapidly in both groups of mice (Fig. 3B,C); normalization of platelet counts, however, was delayed in recipients of AMD3100 infusion-mobilized blood grafts (Fig. 3D).

AMD3100-sensitive HSPC pools

As demonstrated by dose-response curves (Fig. 1A), the total size of the AMD3100-bolus sensitive pools is at best a few 1000 CFU-C in the mouse (mobilization over baseline of <1000 CFU-C/mLx2mL of blood). Because of the small numbers, it was not possible to establish a priori from which organ (BM or extramedullary sources) these mobilized cells originate. The presence of small numbers of functional HSPC in extramedullary spaces, including in non-hematopoietic organs, is well documented.[30,31] Splenectomized mice were treated with AMD3100 to address whether the spleen might be the source of the AMD3100-mobilized CFU-C. The mice had the expected markedly increased baseline circulating CFU-C, but mobilized efficiently in response to bolus injections or continuous infusion of AMD3100 (Fig. 4D), ruling out the spleen as the main organ of origin of AMD3100 mobilized HSPC.

Irrespective of the mobilization regime, all circulating HSPC, including those circulating during steady-state hematopoiesis, have been shown to be quiescent. [32,33] Commonly employed mobilizing agents (cytokines or chemotherapy + cytokines) mobilize HSPC with slow, multi-day kinetics, and are associated with proliferation in the marrow prior to the appearance of HSPC in circulation. It was thus unclear, whether quiescence preceded their egress from marrow, or was acquired in the peripheral blood milieu. The rapid kinetics of mobilization by AMD3100 bolus injection, which do not allow for proliferative or cell cycle changes, offered a unique opportunity to address this issue. Cell cycle of circulating or marrow-derived c-kit+ HSPC was tested using standard flow cytometry-based assays (Acridine orange). Like G-CSF- (Suppl. Fig. 1) or chemotherapy+G-CSF-mobilized HSPC,[32,33] the cell cycle status of AMD3100 bolus-mobilized c-kit+ cells was predominantly G₀ (Fig. 5A and Suppl. Fig. 1), in contrast to normal BM c-kit+ cells, approximately 50% of which were non-G₀. Likewise, AMD3100 infusion-mobilized blood c-kit+ cells were predominantly in G₀ (Fig. 5B). Even in mice with significantly enhanced proliferation in BM (Cyclophosphamide-treated G-CSFR^{-/-} mice, with <20% of BM-c-kit+ cells in G₀), almost all AMD3100 bolus-mobilized c-kit+ cells were quiescent (Fig. 5A). These data would suggest that quiescence is a prerequisite for mobilization, or that cycling cells are excluded from egress from marrow. To further extend these observations, to explore whether completion of cell cycling immediately preceded HSPC egress of AMD3100-mobilizable pools, as previously suggested for Cytosan + G-CSF mobilization[34], we carried out partial BrdU labeling experiments. To this end, mice were pulsed intraperitoneally with BrdU, to achieve labeling of HSPC that were cycling during narrow time windows, followed by bolus-injection of AMD3100 3-72 hours thereafter, and analysis of BrdU incorporation in BM and MPB one hour later. Labeling of 20-40% of BM c-kit+ cells was achieved by this approach, but at all tested time points, BrdU labeled c-kit+ cells were underrepresented in AMD3100 bolus-mobilized blood (Fig. 5G). These data invite two possible interpretations, that either AMD3100-responsive pools are slow-cycling relative to non-responsive BM cells, or are out of cycle during the mobilization window.

If these predictions are true, we wanted to test how mobilization by AMD3100 was influenced at times when active regeneration of BM hematopoiesis occurs, especially following stress, and reciprocally, whether the process of regeneration was adversely affected by the mobilization process. Mice were treated with 5-Fluorouracil. 24 hours thereafter, continuous infusion of AMD3100 or control for a total of 8 days was initiated, and hematopoietic

regeneration was observed. Regeneration of all lineages was entirely normal in AMD3100-treated mice, including the characteristic platelet overshoot (Fig. 2D-F). Mobilization with AMD3100 was also tested in another model of hematopoietic stress, during the engraftment period after BM transplantation, to assess whether quiescent, AMD3100 mobilization-amenable pools of HSPC were available during these times. Mice treated with a bolus of AMD3100 8 days following BM transplantation, i.e. during a time of hematopoietic regeneration marked by dramatic expansion and differentiation, responded with approximately normal mobilization in response to a bolus of AMD3100 (Fig. 4A), suggesting that even under these conditions quiescent HSPC reside in AMD3100-sensitive niches and thus mobilization is not impaired. This was not tested at earlier times, when mobilization might have been inhibited, according to previous reports.[35]

All our prior studies regarding AMD3100 responsive pools within BM at homeostasis and after stress were referable to late CFU-C or progenitor cell pools. To what extent true stem cell pools in BM were affected was unclear. Stem cell function is best tested through transplantation experiments, but small differences may be difficult to identify. Therefore, we resorted to an alternative, novel functional assay for long-term repopulating cells recently described for AMD3100 bolus mobilization, which tests the availability of stem cell niches in the unconditioned host through engraftment differences. This availability was correlated with mobilization efficiency.[36] Given the much more efficient mobilization achieved after AMD3100 pump infusion, we reasoned that such effects might be pronounced in mice treated in this fashion. To formally test this, non-irradiated C57Bl/6 mice, fitted 8 days before transplantation with either sham-pumps or AMD3100-infusions, received i.v. transplants of 30×10^6 C57SLJ BM cells within 1 hour of pump removal, i.e. at a time when mobilized cells remained in circulation. Donor-type engraftment was not observed in either group, in spite of substantial HSPC mobilization in the AMD3100-treated recipients, as shown above. Re-transplantation of BM cells from primary recipients to lethally irradiated CD45.2 hosts after 16 weeks, to potentially detect previously suppressed quiescent stem cells in the primary host, likewise did not uncover any primary donor contribution (data not shown). Nevertheless, for definitive answers about these issues and about the effects of AMD3100 infusion in cases of compromised BM in terms of stem cell reserve, further studies are warranted.

AMD3100 mobilizes HSPC through interference with Gi protein dependent signals

As we have previously shown, treatment of mice with the Gi protein inhibitor Pertussis Toxin (PTX) resulted in the egress of HSPC which peaked 3 days after the injection.[37] (PTX)-treated mice treated with a bolus of AMD3100 do not show additional mobilization above the level seen with PTX alone (Fig. 4C). This observation indicates that overlapping signal transduction pathways, i.e. Gi protein-mediated pathways, are blocked in mobilization by PTX and AMD3100.

To test whether G-CSF receptor signaling was in any way involved in mobilization by AMD3100, G-CSFR^{-/-} mice were treated with AMD3100 boli.[38] Mobilization of the expected efficiency was observed in the G-CSFR^{-/-} mouse, both when tested during steady-state and during the massive proliferation induced by Cyclophosphamide treatment 5 days prior (Fig. 4C).[39] These data reinforce the point that AMD3100 mobilization is independent of G-CSF signaling, and may also suggest a potential of AMD3100 to be useful for G-CSF refractory donors.

DISCUSSION

Because of the protracted kinetics of G-CSF mobilization and the multiple cellular and molecular targets, analysis of the molecular events of HSPC mobilization has remained a challenge. In contrast to G-CSF, the rapid mobilization kinetics of single-bolus administration

of AMD3100, with peaks reached within an hour of i.p. injection in the mouse, is a useful tool to explore mechanisms of HSPC retention and mobilization, as well as properties of AMD3100-mobilizable pools in the BM, and general properties of mobilized HSPC. However, as a single-agent HSPC mobilizing strategy, single-bolus injection of AMD3100 is relatively inefficient, at least in mice, which may limit its usefulness when large numbers of transplantable cells are needed. As shown, mobilization by AMD3100 bolus is short-lived. We reasoned that this might either be the result of a short biological half-life of AMD3100, or it might indicate that HSPC are rapidly desensitized to AMD3100. Repeated injections resulted in the same magnitude of mobilization after each injection (without accumulation, at least at the intervals tested), which argued in favor of the first hypothesis. Together, these observations provided the rationale for testing the effect of continuous infusion. If desensitization was responsible for the short duration of HSPC mobilization after AMD3100 bolus, then enhanced mobilization should not occur during AMD3100 infusion, or it would have been short-lived. However, the data presented here for continuous infusion of AMD3100 demonstrate superb HSPC mobilizing potency. Further, CFU-C are mobilized by AMD3100 infusion with remarkably slow kinetics, possibly because the replenishment of finite AMD3100 sensitive pools from other marrow sites may be rate-limiting. The fact that a maximum number of circulating CFU-C is reached after 3-4 days, likely indicates that at this number equilibrium is reached between HSPC leaving BM and HSPC being cleared from circulation. Clearance of circulating CFU-C after discontinuation of AMD3100 infusion is extremely rapid, similar to that after bolus mobilization. This observation further strengthens our hypothesis that the efficient mobilization after continuous infusion of AMD3100 predominantly reflects the prolonged presence of biologically active AMD3100, i.e. overcomes the short functional half-life of AMD3100 in vivo. Additional effects of long-term CXCR4 blockade on HSPC proliferation [40] or on the hematopoietic microenvironment can, however, not be definitively discounted.

The high yield of circulating HSPC also allowed functional studies of AMD3100 mobilized cells to be pursued. We demonstrate that AMD3100 mobilized HSPC have (in contrast to HSPC migrating in the presence of AMD3100 in vitro) good migratory properties in response to SDF-1. As these and other data indicate, the interaction between in vivo applied AMD3100 and mobilized HSPC is transient, since in vitro sensitivity to SDF-1 was rapidly restored to normal. This observation is likely due to both receptor recycling and decay. Consequently, the superior efficiency of marrow homing of AMD3100 mobilized HSPC compared to BM-derived HSPC was not surprising; it was similar to G-CSF mobilized HSPC.[20] Of interest, overexpression of CXCR4 caused significant increase in mesenchymal cell homing to BM and spleen in irradiated recipients.[41] The functional competency of AMD3100 mobilized cells is also indicated by their repopulation kinetics compared to G-CSF MPB. Taken together, these data indicate that AMD3100 mobilized blood HSPC fulfill all criteria of transplantable cells and should therefore be suitable for clinical application, even though platelet recovery was delayed relative to G-CSF mobilized grafts, which may be a concern.

Now that potent alternatives to G-CSF have been described, the selection of the mobilizing agent can be based on desired properties of the mobilized cells, or on the avoidance of potential adverse effects of the mobilizing agent. G-CSF has been associated with pronounced neutrophilia and the potentially fatal neutrophil activation syndrome in certain donors, which would not be expected after AMD3100.[42,43] G-CSF also induces a pronounced immunomodulation, which triggers relapses in donors with autoimmune diseases.[8,9] Thus donors with sickle cell anemia or autoimmune disease might benefit from mobilization with AMD3100. In the allogeneic transplant setting, G-CSF-mobilized transplants have been associated with improved anti-leukemia effects, but an increased risk for chronic GvHD for the recipients, so much that BM HSPC have become the preferred donor cell source in situations where the risk of chronic GvHD outweighs potential graft-versus-leukemia effects, such as in transplantation for non-malignant illness.[10,44] Recent data suggest that significant

immunomodulation is not induced by AMD3100, suggesting that AMD3100-mobilization of HSPC might be a safe alternative to BM harvest.[45] Of interest, recent data suggest that in autologous HSCT for leukemia, an increased risk of relapse is associated with G-CSF mobilized HSPC than with BM-HSPC.[46]

The observation of normal hematopoietic regeneration in AMD3100-treated recipients of 5-FU suggests that AMD3100 does not influence proliferation of HSPC, calling into question proposed trophic and/or survival enhancing roles of CXCR4/CXCL12 signals for hematopoietic cells,[47] at least during the time of hematopoietic regenerative stress. Moreover, we observed adequate mobilization by AMD3100 in a G-CSF resistant host (G-CSFR^{-/-}). This observation greatly strengthens the previously presented view that specifically individuals with less than optimal responses to G-CSF may benefit from AMD3100 as a mobilizing agent. Similar data were recently demonstrated for Fanconi-mice, which are also refractory to G-CSF.[48]

The quiescence of G-CSF mobilized HSPC was repeatedly documented.[32,33,49] Interpretation of these data was hampered by the slow accumulation of HSPC in blood during G-CSF mobilization. Thus it was not clear whether quiescence was a prerequisite for egress from marrow, or whether quiescence could be acquired due to exposure to the peripheral blood, away from the cytokine-rich BM milieu. Performing cell cycle studies on AMD3100 mobilized cells has the significant advantage over similar studies with G-CSF that the short time between injection of AMD3100, egress from marrow, harvest and analysis does not allow for changes in cell cycle. In other words, the cell cycle status of HSPC retrieved from blood of AMD3100 bolus-mobilized mice likely represents the status of exiting cells at the time of mobilization. As shown here, AMD3100 almost exclusively mobilizes quiescent HSPC, i.e. quiescence is a prerequisite for mobilization by AMD3100. However, does recent completion of cell cycle preferentially target HSPC for egress from marrow, as earlier studies[34] might imply? Wright et al. previously demonstrated that after feeding mice BrdU for several days (resulting in labeling of almost all BM cells), Cy+G-CSF mobilized HSPC were non-cycling, but had incorporated BrdU.[34] Since virtually all HSPC in BM stained positive for BrdU, these studies could not discriminate at what time point after completion of cell cycle HSPC are mobilized. Our studies differ from the prior studies in their experimental design, i.e. pulsing mice with BrdU 3 hours - 3 days before mobilization, thereby achieving partial labeling (20-40%) of BM c-kit⁺ cells, and by employing an agent with fast mobilization kinetics. Under these conditions, very few BrdU labeled c-kit⁺ cells were mobilized by AMD3100, well below the expected proportion based on BrdU labeling in BM c-kit⁺ cells, indicating that AMD3100 targets a pool of non-cycling HSPC, in which recently cycled cells are underrepresented. These observations suggest that the exchange between proliferating pools and AMD3100 sensitive pools is slow during homeostasis.

Our experiments concluded that marrow is the main source of AMD3100 mobilized HSPC. But where in the BM are the AMD3100 mobilizable cells located? Because of the paucity of information regarding localization of any mobilizable pool by any mobilizing agent and the absence of significant quantitative changes in BM after AMD3100 (even after continuous infusion), it is hard to pursue this question directly. As AMD3100 mobilizes quiescent HSPC, and does so with rapid kinetics, one would have thought that the rapid kinetics of AMD3100-bolus mobilization may not allow for migration across significant distances, thereby suggesting that the mobilizable pool may be located in proximity to the BM-blood barrier, near the BM sinusoidal endothelium. However, if this is the case, this would imply that quiescent HSPC reside in “vascular” regions, considered by some the niche for proliferative HSPC pools. Nevertheless, such a conclusion is consistent with the findings of Kiel et al. showing localization of the majority of the quiescent stem cell pool around sinusoids.[50]

The role of the CXCR4/CXCL12 axis in HSPC mobilization has been extensively studied. Thus agonists, such as MetSDF-1,[51] virally overexpressed CXCL12 [52] or small-molecule CXCR4 agonists,[53] as well as CXCR4 antagonists [11] mobilize HSPC, presumably by the same mechanism, i.e. de-sensitization of CXCR4 to SDF-1 expressed on BM extra-cellular matrix and stromal cells. These observations on the one hand suggested interference with the CXCR4/CXCL12 signaling as a targeted approach for HSPC mobilization, on the other hand implied a role for CXCR4/CXCL12 as a marrow retention factor for HSPC. Data from mice largely support this hypothesis, in that several studies demonstrated that HSPC deficient for CXCR4 are continuously released from marrow.[54,55] Several lines of evidence indicate that interference with SDF-1/CXCR4 signaling is also instrumental for mobilization by G-CSF or Cyclophosphamide+G-CSF.[56] Thus, during G-CSF mobilization, CXCR4 is down-regulated and SDF-1 in BM is degraded and/or cleaved.[3,4,57,58] In fact, a recent report suggests that the CXCR4/CXCL12 axis is the critical pathway of G-CSF mediated mobilization.[59] In this context the synergistic mobilization response to a bolus of AMD3100 given after optimal G-CSF stimulation, described by us in agreement with previous reports, [14] is surprising. These data may indicate that the interference of G-CSF with the CXCR4/CXCL12 pathway is rather incomplete and that additional pathways are targeted during G-CSF mobilization.

In summary, the studies reported here reaffirm and extend studies about mobilization with AMD3100, and establish several novel findings: We are presenting continuous infusion of the CXCR4 antagonist AMD3100 as a potent, G-CSF free mobilization scheme, generating cell populations fulfilling all requirements of transplantable cells, including adequate stem cell frequency, efficient homing and good engraftment properties. Our data suggest the presence of a finite, but rapidly replenished pool of AMD3100 mobilization sensitive CFU-C at steady-state. This AMD3100-sensitive HSPC pool is quiescent relative to BM-resident HSPC and is located in the BM, likely in vascular regions, or the “endothelial niche”.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Mobilization of progenitor cells and stem cells by AMD3100

A: Mobilization by AMD3100 bolus injection: Mobilization kinetics (left): Mice received i.p. bolus injections of AMD3100 (100 µg) at 0 hrs. CFU-C were enumerated by colony assay at the indicated times. The number of circulating CFU-C peaked after 1 hour and returned to normal within 4 hours. Asterisks indicate statistically significant differences compared to baseline. CFU-C after 1 hour were statistically significantly higher than at all other time points. Dose-response curve (right): Mice received i.p. injections of the indicated dose of AMD3100. CFU-C were enumerated by colony assay one hour after injection. Mobilization peaked after a dose of 100 µg, equivalent to ca. 4 mg/kg. Asterisks indicate statistically significant differences compared to baseline.

B: Response to repeated AMD3100 stimulation: Mice received 100 µg boli of AMD3100 ([AMD b]) every 12 hours for 6 successive days (left) or at 0 and 4 hours (nadir according to Fig 1A, right), as indicated by arrows. At the time points indicated by grey bars, circulating CFU-C were enumerated (mean+SEM). Mobilization of the same magnitude was observed at all time points, without accumulation of circulating CFU-C. (All times significantly greater than baseline, as indicated by asterisks)

C: Continuous infusion of AMD3100 [AMD i] dramatically improves mobilization efficiency; kinetics of mobilization with [AMD i]: During continuous infusion of AMD3100, CFU-C accumulated relatively slowly, achieving only half-maximal numbers after 24 hours, while clearance from blood after discontinuation of infusion was very rapid. This observation illustrates the previously demonstrated short biologic half-life of AMD3100 in vivo. Asterisks indicate CFU-C numbers in excess of baseline.

D: Efficacious mobilization by continuous infusion of AMD3100: CFU-C contents in BM and MPB: Mobilization with [AMD i] was equivalent to approximately 3-4% of the total CFU-C content of a mouse and was not accompanied by a significant reduction of CFU-C content in femurs. G-CSF infusion primed BM [G-CSF i] contained reduced numbers of CFU-C, fewer than can be explained by mobilization alone. The highly efficacious mobilization with G-CSF infusion + AMD3100 bolus [G-CSF i + AMD b] resulted in mobilization of more than two femur equivalents and was accompanied by an additional reduction in CFU-C contents in BM relative to G-CSF infusion alone.

E: Quantities administered during AMD3100 infusion are saturating: Mice received [AMD b], [AMD i], [AMD i] followed by [AMD b] on day 5, or [G-CSF i]. Where applicable, circulating CFU-C were enumerated 1 hour after [AMD b]. After [AMD i], CFU-C/mL were 10-fold increased over [AMD b] ($p<0.001$), and 30- to 50-fold increased over baseline, to numbers which compare favorably to those achieved with G-CSF. Administration of [AMD b] after [AMD i] did not result in additional mobilization compared to [AMD i], suggesting that satiating doses of AMD3100 were administered. Asterisks indicate statistically significantly increased CFU-C of [AMD i] relative to [AMD b] and [G-CSF] relative to [AMD i].

F: Mobilization of CRU: CRU frequency in [AMD b] or [AMD i] mobilized blood was compared by limiting dilution transplantation assays. Groups of 8-10 lethally irradiated mice (CD45.2) received transplants of 250,000 normal BM cells (CD45.2) plus the indicated volume of mobilized blood (CD45.1). Engraftment levels of CD45.1 cells were quantified 16 weeks after transplantation; mice with $\geq 1\%$ CD45.1 cells were considered positive for mobilized blood engraftment. Dose dependent engraftment derived from mobilized blood was observed, (AMD b = black diamonds, AMD i = grey squares) (**left**). Mean CRU frequency was 77/mL for [AMD b] blood, 273/mL for [AMD i] blood (**right**) (mean+S.D.). This difference was statistically significant.

Fig. 2. Effect of AMD3100 on HSPC in vitro migration and adhesion; effect of AMD3100 infusion on myelosuppression by 5-Fluorouracil

A: c-kit+ BM cells were isolated and incubated in vitro with AMD3100 (100 μ g/mL) or medium, followed by transwell migration towards medium (grey bars) or SDF-1 (100 ng/mL, black bars) for three hours. As previously shown, AMD3100 incubation almost completely blocked migration towards SDF-1 (asterisk indicate statistically significant differences compared to not AMD3100 treated control cells).

B: [AMD i] mobilized peripheral blood cells (grey) or steady-state BM cells (black) were allowed to migrate towards SDF-1 (100 ng/mL) for four hours, followed by incubation of

migrated cells in colony assays. The fraction of migrated CFU-C was similar for [AMD i] MPB and ssBM.

C: c-kit⁺ cells were treated in vitro with AMD3100 (100 µg/mL) for 20 minutes, followed by incubation on RetroNectin for 2 hours. Adhering cells were quantified by Crystal Violet staining. There was no difference in adhesion to RetroNectin between AMD3100 treated and untreated c-kit⁺ cells.

D-F: No evidence for hematopoietic suppression by AMD3100-infusion: Mice received single injections of 250 mg/kg of 5-fluorouracil, followed by continuous infusion of [AMD i] or [PBS/BSA] for a total of 8 days, starting 24 hours after 5-FU. Hematopoietic regeneration was monitored. The recovery of hematocrit (Hct, **D**), white cell count (WBC, **E**) or platelet count (Plt, **F**) was no different in control and AMD3100 treated animals.

Fig. 3. BM homing and engraftment of AMD3100-mobilized HSPC in lethally irradiated recipients

A: Recipients received lethal irradiation conditioning, followed by transplantation of [AMD i] or [G-CSF i] mobilized peripheral blood cells ([AMD i MPB] or [G-CSF i MPB]), of BM cells from these mobilized animals ([AMD i BM] or [G-CSF i BM]), or of BM cells from untreated controls ([ssBM]). 20 hours after transplantation, BM homing was quantified relative to the number of injected CFU-C. Homing of [AMD i MPB] or [G-CSF i MPB] was significantly superior to that of [ssBM], while homing of [AMD i BM] or [G-CSF i BM] was significantly lower than that of [ssBM].

B, C, D: Engraftment kinetics of mobilized HSPC: Lethally irradiated hosts received transplants of 100 μ l AMD3100 infusion-mobilized [AMD i] or G-CSF-mobilized [G-CSF] peripheral blood. CBC were followed as indicated. All recipients survived with this cell dose. Engraftment kinetics of erythrocytes (**B**) and leukocytes (**C**) were the same for both groups, while normalization of platelet counts was delayed in [AMD i MPB] recipients (asterisks) (**D**).

Fig. 4. CFU-C mobilization by AMD3100 in different mouse models

A: CFU-C mobilization by AMD3100 during hematopoietic stress: 8 days after transplantation of 5×10^6 normal BM c-kit⁺ cells, mice received injections of PBS/BSA or [AMD b]. [AMD b] treated mice responded with significant CFU-C mobilization of at least similar magnitude as during steady-state. This difference from baseline was statistically significant.

B: CFU-C mobilization by AMD3100 in G-CSFR^{-/-} mice: Untreated G-CSFR^{-/-} mice (steady-state, left), or G-CSFR^{-/-} mice treated 5 days prior with Cyclophosphamide (right), responded to [AMD b] with significant CFU-C mobilization from very low baselines, as indicated by asterisks.

C: CFU-C mobilization by AMD3100 in mice treated with a Gi protein inhibitor: As described, Pertussis Toxin (PTX) mobilizes CFU-C. [AMD b] did not mobilize additional CFU-C, indicating that PTX and AMD3100 mobilize by the same molecular mechanism.

D: CFU-C mobilization by AMD3100 in splenectomized mice: Splenectomized mice showed the expected elevation in baseline CFU-C. Mobilization by [AMD b, left] or [AMD i, right] was at least as efficient as in not splenectomized animals, indicating that AMD3100 mobilized cells do not originate in the spleen.

Fig. 5. Cell cycle status of HSPC mobilized by AMD3100

A-F: AMD3100 mobilized HSPC are quiescent: Five days after Cyclophosphamide-treatment, G-CSFR^{-/-} mice received [AMD b] followed by exsanguination 1 hour later (**A**), or untreated wild-type mice were exsanguinated after a 5-day course of [AMD i] (**B**). BM cells from similarly treated (PBSBSA injection or sham-pumps, respectively) donors were used for comparison in each case. c-kit⁺ cells were isolated by flow sorting, and cell cycle status was analyzed by Acridine Orange staining. MPB c-kit⁺ cells were overwhelmingly in G₀, in contrast to BM c-kit⁺ cells. Asterisks indicate statistical significance of the difference relative to BM. **C,D** show a representative DNA/RNA dot plot, **E,F** a representative DNA histogram

from one of the samples represented in D, with mobilized blood c-kit⁺ cells on the left (**C,E**), steady-state BM c-kit⁺ cells on the right (**D,F**).

G: Mice pulsed with BrdU 2, 23 or 47 hours before mobilization, or not pulsed with BrdU, received [AMD b] followed by exsanguination one hour later. While 3, 24 or 48 hours after BrdU injection 20->40% of BM c-kit⁺ cells were labeled with BrdU, i.e. had entered S-phase, the frequency of BrdU labeled cells among mobilized peripheral blood c-kit⁺ cells was very low. Asterisks indicate statistically significant differences to BM labeling.

Expression of surface molecules on c-kit+ cells

Mice were treated with a single bolus of AMD3100, and mobilized blood was harvested one hour later, or with G-CSF infusions or AMD3100 infusions for 5 days. Expression of surface molecules was analyzed by gating on c-kit+ cells and simultaneous analysis of expression of the respective surface marker, using directly conjugated antibodies. For each surface marker, % positive cells relative to isotype control and mean fluorescence intensity (MFI) among c-kit+ was evaluated. Overall, expression of adhesion molecules on mobilized c-kit+ cells, irrespective of the mobilization regime, was lower than on BM-resident c-kit+ cells. CD26 and CXCR4 were dramatically reduced on G-CSF-mobilized c-kit+ cells, while expression was preserved on AMD3100 mobilized cells.

	CD49b	CD49d	CD49e	CD49f	CD29	CD62L	CD26	CXCR4
ssBM								
% expression±SEM	12±2	99±0	89±0.3	89±1	100±0	49±1	43±2	41±2
MFI±SEM	13±2	2732±140	93±4	135±3	364±4	40±12	26±2	18±0.3
G-CSF infusion								
% expression±SEM	27±15	94±0.3	66±9	72±14	99±1	74±2	2±1	2±1
MFI±SEM	19±7	38±2	24±4	56±26	109±33	36±6	8±0.3	5±0.3
AMD3100 infus.								
% expression±SEM	21±8	93±3	50±6	58±3	85±3	50±5	55±5	47±7
MFI±SEM	20±3	129±10	34±5	85±15	158±11	64±4	31±2	24±2
AMD3100 bolus								
% expression±SEM	19±2	94±1	46±3	56±3	85±4	58±4	55±1	42±5
MFI±SEM	16±1	216±90	31±2	108±4	130±4	95±13	29±1	57±19