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Mobilized peripheral blood has become the primary source of hematopoietic stem and progenitor cells (HSPCs) for stem cell transplantation, with a 5-day course of granulocyte colony-stimulating factor (G-CSF) as the most common regimen used for HSPC mobilization. The CXCR4 inhibitor plerixafor is a more rapid mobilizer, yet not potent enough when used as a single agent, thus emphasizing the need for faster acting agents with more predictable mobilization responses and fewer side effects. We sought to improve hematopoietic stem cell transplantation by developing a new mobilization strategy in mice through combined targeting of the chemokine receptor CXCR2 and the very late antigen 4 (VLA4) integrin. Rapid and synergistic mobilization of HSPCs along with an enhanced recruitment of true HSCs was achieved when a CXCR2 agonist was coadministered in conjunction with a VLA4 inhibitor. Mechanistic studies revealed involvement of CXCR2 expressed on BM stroma in addition to stimulation of the receptor on granulocytes in the regulation of HSPC localization and egress. Given the rapid kinetics and potency of HSPC mobilization achieved by the VLA4 inhibitor and CXCR2 agonist combination in mice compared with currently approved HSPC mobilization methods, the combination represents an exciting potential strategy for clinical development in the future.

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Targeting VLA4 integrin and CXCR2 mobilizes serially repopulating hematopoietic stem cells

Darja Karpova,1,2 Michael P. Rettig,1 Julie Ritchey,1 Daniel Cancilla,1 Stephanie Christ,1 Leah Gehrs,1 Ezehilarasi Chendamarai,1 Moses O. Evbouwuan,3 Matthew Holt,1 jingzhu Zhang,1 Grazia Abou-Ezzi,1 Hamza Celik,1 Eliza Wiercinska,1 Wei Yang,5 Feng Gao,6 Linda G. Eissenberg,1 Richard F. Heier,2 Stacy D. Arnett,7 Marvin J. Meyers,2 Michael J. Prinsen,7 David W. Griggs,7 Andreas Trumpf,2 Peter G. Ruminski,1,7 Dwight M. Morrow,8 Halvard B. Bonig,4,8 Daniel C. Link,1 and John F. DiPersio1

1Division of Oncology, Department of Medicine, Washington University School of Medicine, St. Louis, Missouri, USA. 2Division of Stem Cells and Cancer, German Cancer Research Center (DKFZ) and DKFZ-ZMBH Alliance, Heidelberg, Germany. 3Oakland University William Beaumont School of Medicine, Rochester, Michigan, USA. 4German Red Cross Blood Service and Institute for Transfusion Medicine and Immunohematology of the Goethe University, Frankfurt, Germany. 5Genome Technology Access Center, Washington University, St. Louis, Missouri, USA. 6Division of Public Health Sciences, Department of Surgery, Washington University School of Medicine, St. Louis, Missouri, USA. 7Center for World Health and Medicine, Saint Louis University, St. Louis, Missouri, USA. 8Magenta Therapeutics, Cambridge, Massachusetts, USA. 9University of Washington, Department of Medicine/Hematology, Seattle, Washington, USA.

Mobilized peripheral blood has become the primary source of hematopoietic stem and progenitor cells (HSPCs) for stem cell transplantation, with a 5-day course of granulocyte colony-stimulating factor (G-CSF) as the most common regimen used for HSPC mobilization. The CXCR4 inhibitor plerixafor is a more rapid mobilizer, yet not potent enough when used as a single agent, thus emphasizing the need for faster acting agents with more predictable mobilization responses and fewer side effects. We sought to improve hematopoietic stem cell transplantation by developing a new mobilization strategy in mice through combined targeting of the chemokine receptor CXCR2 and the very late antigen 4 (VLA4) integrin. Rapid and synergistic mobilization of HSPCs along with an enhanced recruitment of true HSCs was achieved when a CXCR2 agonist was coadministered in conjunction with a VLA4 inhibitor. Mechanistic studies revealed involvement of CXCR2 expressed on BM stroma in addition to stimulation of the receptor on granulocytes in the regulation of HSPC localization and egress. Given the rapid kinetics and potency of HSPC mobilization achieved by the VLA4 inhibitor and CXCR2 agonist combination in mice compared with currently approved HSPC mobilization methods, the combination represents an exciting potential strategy for clinical development in the future.

Introduction
In postnatal mammals, the vast majority of hematopoietic stem and progenitor cells (HSPCs) reside in the protective environment of the bone marrow (BM) (1, 2). Accordingly, only very few HSPCs can be found in the periphery, primarily in blood and spleen, during homeostasis (3). A wide variety of stimuli has been identified that elicits HSPC egress from the marrow, a phenomenon referred to as mobilization (4). Despite its significance and the preclinical discovery of various approaches to lure HSPCs into the circulation during the past 2 decades, the armamentarium of clinical mobilization remains sparse. Thus, granulocyte colony-stimulating factor (G-CSF) is the sole agent approved for mobilization of healthy donors, whereas in patients chemotherapy and the small molecule antagonist of the chemokine receptor CXCR4, AMD3100 (Plerixafor), are approved in conjunction with G-CSF (5).

Limitations of currently available clinical regimens provide the rationale for the ongoing search for alternative mobilization strategies. Importantly, all approaches described to date target at least 1 of the 2 major axes mediating HSPC retention: the chemokine receptor CXCR4 or the integrin very late antigen 4 (VLA4) (4). Despite its significance and the preclinical discovery of various approaches to lure HSPCs into the circulation during the past 2 decades, the armamentarium of clinical mobilization remains sparse. Thus, granulocyte colony-stimulating factor (G-CSF) is the sole agent approved for mobilization of healthy donors, whereas in patients chemotherapy and the small molecule antagonist of the chemokine receptor CXCR4, AMD3100 (Plerixafor), are approved in conjunction with G-CSF (5).

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The chemokine receptor CXCR2 is a critical regulator of neutrophil chemotaxis (15). Interestingly, stimulation of CXCR2 signaling (e.g., with its ligands CXCL1, -2, or -8) has been demonstrated to result in rapid HSPC mobilization from the BM even though the receptor is not expressed on HSPCs themselves (16–20). The mechanism of HSPC egress after CXCR2 stimulation remains controversial.

In this study, we explored the potential of combined targeting of VLA4 and CXCR2 signaling as a novel strategy to mobilize HSPCs. In addition to being associated with a rapid and remarkably synergistic HSPC release, administration of a VLA4 antagonist in conjunction with a CXCR2 ligand targeted very primitive, serially repopulating HSPCs with high efficiency. Our observations challenge the notion of limited potential of fast-acting mobilizing agents and are particularly relevant in the context of the ongoing debate about the vascular versus endosteal localization of stem cells (21–23). Moreover, unexpectedly, stromal cells were found to be targeted by CXCR2 stimulation along with neutrophils. This contribution of both stromal and neutrophilic CXCR2 to the regulation of HSPC localization underlies the intricate interplay between nonhematopoietic and mature hematopoietic cells for HSPC maintenance.

Results

Combined targeting of VLA4 and CXCR2 results in augmented HSPC recruitment. We assessed the mobilization efficiency of a VLA4 antagonist alone compared with a CXCR2 agonist alone, as well as their combined effect in vivo. The previously described small-molecule VLA4 inhibitor firategrast (24, 25) along with the naturally occurring truncated form of the CXCR2 ligand Gro-β (tGro-β) (26, 27) were used in the initial experiment. As shown in Figure 1, combined treatment with both agents resulted in increased numbers of circulating WBCs and CFU-Cs of up to 3- and 10-fold, respectively (Figure 1A and B). To circumvent the shortcomings of VLA4-targeting compounds hitherto tested as mobilizing agents, we selected a series of VLA4 antagonists that had been developed based on the structure of the well-known VLA4 inhibitors BIO5192 (28) and firategrast (24, 25). These compounds (CWHM-822, 823, 824, 825, and 842; Figure 1C) were synthesized as previously described (29–34). Properties of the inhibitors were assessed using a colorimetric, cell-free, solid-phase receptor binding assay (SPRA; Supplemental Table 1; supplemental material available online described (29–34)). Properties of the inhibitors were assessed using a colorimetric, cell-free, solid-phase receptor binding assay (SPRA; Supplemental Table 1; supplemental material available online described (29–34)).

We next tested whether the synergism between VLA4 inhibition and CXCR2 stimulation was a compound class as opposed to a compound-specific effect. Therefore, mobilization with BIO5192 and firategrast was tested alongside the new compounds, CWHM-823 and -842. All 4 inhibitors mobilized HSPCs by themselves, whereas the mobilization response was enhanced up to 3- to 10-fold when combined with tGro-β (Figure 1E), suggesting a compound class-specific effect. Firategrast-related CWHM-823 outperformed the BIO5192-related CWHM-842 in vivo and was therefore selected for the majority of our subsequent analyses.

Optimal pharmacokinetics and pharmacodynamics were determined to be associated with subcutaneous administration of the CWHM-823 plus tGro-β mixture (Supplemental Figure 1, A and B). Time and dose-response analysis revealed no increase in mobilization between 3 mg/kg and 15 mg/kg of CWHM-823, whereas peak mobilization was reached approximately 30 minutes after the injection (Supplemental Figure 1C).

Complementary to the testing of different VLA4 inhibitors, stimulation with tGro-β (CXCL2) was compared with that of the alternative CXCR2 ligands CXCL1 (Gro-α) and CXCL8 (IL-8). Again, all 3 agonists induced HSPC mobilization when given alone as well as in combination with CWHM-823 (Figure 1F). To control for specificity of the observed effects, CXCR2-KO mice were included. As expected, CXCR2 ligands alone did not induce mobilization in CXCR2-KO mice. Mobilization with the VLA4 antagonist was higher in absolute numbers yet qualitatively unchanged considering the higher baseline levels of circulating CFU-C (930 CFU-C/ml [BALB/cJ CXCR2-KO] versus 300 CFU/ml [BALB/cJ WT]) at baseline, and 3800 CFU-C/ml [BALB/cJ CXCR2-KO] versus 1300 CFU-C/ml [BALB/cJ WT] mobilized with CWHM-823. Surprisingly, a decrease in mobilization with CWHM-823 was observed in CXCR2-KO mice when CXCR2 ligands were coadministered with the VLA4 antagonist. One possible explanation for this is that the bioavailability of CWHM-823 is reduced upon administration in conjunction with the chemokine as compared with its administration alone (Supplemental Figure 1B). Lack of specificity for the target receptor CXCR2 appears unlikely: in our comprehensive screening of tGro-β against a panel of 348 different G protein–coupled receptors, no cross-reactivity of the chemokine with any receptors other than CXCR2 was found (Supplemental Table 2).

Properties of mobilized cells. Having established that VLA4 inhibition combined with CXCR2 stimulation achieves superior CFU-C mobilization, we compared the repopulating capacity of the grafts mobilized with this new regimen, the single agents, or G-CSF (Figure 2A). For both VLA4 antagonists tested (firategrast and CWHM-823), a significantly increased blood graft–derived contribution in the primary recipients was detected when each antagonist was combined with tGro-β compared with engraftment from blood mobilized by either of the VLA4 antagonists or tGro-β alone (Figure 2B). More importantly, despite higher numbers of CFU-Cs mobilized by G-CSF (Supplemental Figure 2 and data not shown), the concentration of repopulating units in the blood of mice mobilized with VLA4 antagonist plus Gro-β was equal to that of G-CSF. Analysis of secondary recipients (i.e., evidence of serially repopulating units) confirmed virtually identical mobilization potency within the immature HSC fraction for G-CSF and VLA4 antagonist plus tGro-β (Figure 2C). Moreover, in a model of diabetes-associated poor mobilization (10, 35, 36) generated by exposure of mice to the pancreas toxic agent streptozotocin, mobilization was better preserved with VLA4 antagonist plus Gro-β combination as compared with G-CSF (7200 CFU-C/ml in diabetic compared with 22,200 CFU-C/ml mobilized in healthy mice with G-CSF; 9000 compared with 6350 CFU-C/ml with CWHM-823 plus tGro-β, Supplemental Figure 2).

We then performed gene expression profiling of differentially mobilized HSPCs. LSK cells isolated from the blood of mice treated with AMD3100 (1 hour), G-CSF (5 days), or CWHM-823 plus tGro-β (30 minutes) along with LSK cells from BM of untreated
mice were examined using microarray analysis. A high degree of similarity between the analyzed LSK types was found, with AMD3100-mobilized LSK cells being the only group clustering somewhat separately from the other 3 using principal component analysis (PCA, Figure 2D). In comparison to BM, LSK cells mobilized by CWHM-823 plus tGro-β had only 45 genes with significantly different expression levels (Supplemental Table 3).

CWHM-823 plus tGro-β versus G-CSF had only 53 such genes (Supplemental Table 4). By contrast, 694 genes were significantly up- or downregulated between AMD3100 and CWHM-823 plus tGro-β–mobilized LSK cells despite their very similar kinetics of mobilization (Supplemental Table 5). The close relationship between CWHM-823 plus tGro-β–mobilized LSK cells, BM-resident LSK cells, and G-CSF LSK cells was further evident upon...
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Figure 2. Properties of mobilized HSPCs. (A) Schema for analyzing competitive repopulating capacity. Blood (10 μl) from CD45.1+ donors (BALB/cJ, n = 2–3) mobilized with G-CSF (9 doses every 12 hours, 100 μg/kg per dose), tGro-β (2.5 mg/kg, 15 minutes after s.c. injection), VLA4 antagonist fircatragest (100 mg/kg, 1 hour after s.c. injection), and CWHM-823 (3 mg/kg, 1 hour after s.c. injection), or the combination of tGro-β and VLA4 antagonist (dosed as indicated for single injections, 30 minutes after simultaneous injection) was mixed and transplanted into lethally irradiated primary CD45.2+ hosts (BALB/cJ, n = 8–10 recipients). (B) Percent CD45.1+ donor cells within the CD45.1–CD3+ compartment of blood was evaluated 20 weeks after transplantation. Data are mean ± SEM, n = 8–10. ***P < 0.001 compared with VLA4 antagonist alone/combined with tGro-β alone. (C) BM from the primary recipients was harvested, pooled, and transplanted into lethally irradiated secondary recipients (CD45.2–BALB/cJ, 2.5 × 105 per recipient). The percentage of donor-derived cells in the blood of secondary recipients 18 weeks after transplantation is shown. Data are mean ± SEM, n = 5 and are not significantly different. *P < 0.05. (D–F) LSK cells were sorted from the blood of DBA/2J mice mobilized with G-CSF (9 doses every 12 hours, 100 μg/kg per dose, n = 3). AMoD100 (5 mg/kg, 1 hour after injection, n = 3), or CWHM-823 plus tGro-β (3 mg/kg and 2.5 mg/kg, 30 minutes after simultaneous s.c. injection, n = 3). LSK cells from AMoD100–mobilized BM were included as control. Total RNA from sorted cells was subjected to microarray expression analysis. (D) Principal component analysis (PCA) of mRNA expression of different LSK species. (E) Corresponding hierarchical clustering map. (F) Normalized expression values for the genes Nr4A1-3. Each source/treatment group included n = 3 samples (pooled from up to 6 donors). Statistical comparisons were made using ANOVA, followed by step-down Bonferroni’s adjustment for multiple comparisons.

LA4 antagonist (CWHM-823, 45 minutes prior to tGro-β administration) did not alter subsequent tGro-β-induced mobilization (Figure 3A). In contrast, albeit lower than when both compounds were given simultaneously, significantly elevated numbers of circulating CFU-Cs were detected in CWHM-823-mobilized mice that had been pretreated with tGro-β as compared with PBS. These findings pointed toward CXCR2 targeting as the priming event.

Within the hematopoietic compartment, CXCR2 expression was detected almost exclusively in granulocytes, and specifically HSCs themselves were CXCR2-negative (Supplemental Figure 4). Therefore, as expected, when granulocytes were depleted from the circulation (Figure 3, B and C), mobilization responses to tGro-β alone as well as to the combination of CWHM-823 plus tGro-β were completely blunted (Figure 3D).

In order to distinguish between hematopoietic-intrinsic versus nonhematopoietic contributions for mobilization with CWHM-823 plus tGro-β, chimeraic animals were generated by transplanting CXCR2-KO or WT BM cells into WT recipients and vice versa, WT cells into CXCR2-KO or WT recipients (Figure 4A). Following reconstitution, mice were mobilized with CWHM-823 alone, tGro-β alone, or the combination. As expected, mobilization with the VLA4 antagonist alone was similar in both hematopoietic and stromal CXCR2 knockouts (Figure 4B). No HSPC mobilization with tGro-β alone was observed in WT recipients reconstituted with CXCR2-KO BM (Figure 4C, first and second bars from left). Surprisingly, CXCR2-KO recipients engrafted with the WT BM also demonstrated attenuated mobilization (Figure 4C, fifth and sixth bars from left), indicating an additional contribution of nonhematopoietic (stromal) CXCR2 to the tGro-β–induced HSPC egress. Moreover, the combination of CWHM-823 and tGro-β mobilized HSPCs with almost equal potency in WT recipients of CXCR2-KO and WT BM (Figure 4D, comparing second and fourth bars from left). Mobilization of similar magnitude was also detected in CXCR2-KO and WT recipients reconstituted with WT BM. Stimulation of either hematopoietic or stromal CXCR2 was therefore sufficient to elicit synergistic mobilization when combined with inhibition of VLA4 signaling.

Expression and functional role of CXCR2 in endothelial cells (ECs) has been described (38, 39). Our previous studies indicated the absence of CXCR2 expression in CXCL12-abundant reticular cells (CARs, another major cellular player involved in HSPC retention) (40, 41). We therefore tested whether CXCR2 ablation restricted to ECs would lead to changes in mobilization responses. As shown in Figure 4E, mobilization with all 3 regimens (CWHM-823, tGro-β, and CWHM-823 plus tGro-β) was decreased in CXCR2 EC-KO recipients compared with the control group. Most pronounced was the effect observed with tGro-β alone.

Role of cellular adhesion. Our studies suggested that stimulation of CXCR2 on either granulocytes or stroma was sufficient to recruit as many HSPCs into the circulation as when the target receptor was present in both compartments (Figure 4). This led us to investigate the role of adhesive cross-stimulatory interaction between neutrophils and stroma, likely initiated through exposure to the chemokine. Beta 2 integrins LFA1 (ITGAL/ITGB2, CD11a/CD18) and Mac1 (ITGAM/ITGB2, CD11b/CD18) are well-known crucial mediators of neutrophil adhesion to vasculature (42, 43). Both have been shown to become activated after CXCR2 stimula-
tion (44–46) and have been previously implicated in CXCR2- and G-CSF–triggered mobilization (19, 47, 48).

At baseline, no significant hematological changes were found in mice lacking LFA1 (CD11a-KO) or Mac1 (CD11b-KO) except for a slight increase in circulating neutrophils in CD11a knockouts (Figure 5, A–C). Mobilization of CFU-C with CWHM-823 was approximately doubled in CD11a- or CD11b-deficient mice, whereas both strains’ response to tGro-β alone was the same as in WT mice, despite a dramatic neutrophilia induced in the CD11a-KO mice in response to tGro-β (Figure 5B). The response to the combination treatment was significantly attenuated (Figure 5C).

Role of proteases. Considering the fact that CXCR2 is not expressed on the surface of the HSPCs themselves, we next addressed the question of the molecular crosstalk initiated by CXCR2 stimulation and how it enables HSPC egress. Previous reports aiming to elucidate the mechanism of tGro-β and AMD3100...
plus tGro-β-induced HSPC release suggested critical involvement of the matrix metalloprotease 9 (MMP9) (49–52). Therefore, we compared mobilization with the combination of CWHM-823 plus tGro-β against AMD3100 plus tGro-β as well as single agent treatments in MMP9-KO mice on both FVB and C57BL/6 (B6) background (Supplemental Figure 5, A and B). In addition, a pharmacologic model in the form of a broad-spectrum MMP inhibitor, batimastat, was employed (Supplemental Figure 5C). Baseline circulating HSPC numbers or AMD3100-induced mobilization were not affected by the genetic MMP9 deficiency or by the MMP blockade. CWHM-823–induced mobilization was equally potent in FVB WT and FVB MMP9-KO mice and was not affected by batimastat treatment.

Figure 4. CXCR2 expression in both the hematopoietic and nonhematopoietic (stromal) compartment contributes to tGro-induced mobilization. (A) Hematopoietic-specific CXCR2-KO mice were generated by transplanting lethally irradiated CD45.1+ recipients (BALB/c) with CD45.2+ BM cells isolated from CXCR2-KO mice (BALB/c, 1 × 10^6 cells per recipient, n = 3 BM donor mice). A control group was reconstituted with CD45.2+ WT BM cells (BALB/c, 1 × 10^6 cells per recipient, n = 3 BM donor mice). Stromal-specific knockouts were generated by transplanting CD45.1+ WT BM cells (BALB/c, 1 × 10^6 cells per recipient, n = 3 BM donors) into CXCR2-KO mice (CD45.2). In the corresponding control group, WT CD45.2+ recipients were transplanted with the WT CD45.1+ BM graft. (B–D) Three months after transplantation, circulating HSPC numbers (Lin−Kit+ cells) were assessed in the different recipients at baseline and following mobilization with CWHM-823 alone (3 mg/kg, 1 hour after s.c. injection, B), tGro-β alone (2.5 mg/kg, 15 minutes after s.c. injection, C), and both agents combined (dosed as indicated for separate treatments, 30 minutes after simultaneous s.c. injection, D). Each bar is mean ± SEM, n = 4–5. ***P < 0.001, **P < 0.01, *P < 0.05. (E) Lethally irradiated CXCR2fl/fl Cdh5Cre+ hosts (C57BL/6 background, CD45.2+) were reconstituted with syngeneic WT CD45.1+ BM (3 × 10^6 cells per recipient) to generate EC-specific knockout recipients. In the control group, CXCR2fl/+Cre+ and CXCR2+/+ Cre+ mice were used as recipients. Three months after transplantation, circulating HSPC (CFU-C) numbers were quantified at baseline and following mobilization with CWHM-823 alone (3 mg/kg, 1 hour after s.c. injection), tGro-β alone (2.5 mg/kg, 15 minutes after s.c. injection), and both agents combined (dosed as indicated for separate treatment, 30 minutes after simultaneous s.c. injection). Each bar is mean ± SEM, n = 6. Statistical comparisons were made using ANOVA, followed by step-down Bonferroni’s adjustment for multiple comparisons. Logarithm transformation was performed for the data in D and E.
of MMP9 in CWHM-823 plus tGro-β-associated augmented mobilization. Rather, they suggest differences in the mechanism underlying mobilization induced by combining CXCR2 stimulation and VLA4 blockade compared with CXCR2 activation in conjunction with CXCR4 blockade.

Discussion

In the current study, a novel mobilization strategy, CXCR2 stimulation combined with inhibition of the VLA4 integrin, was investigated and found to result in a rapid, synergistic, and highly efficient recruitment of HSPCs into the circulation. Implementation of VLA4 antagonists with improved properties allowed for comprehensive testing and optimization of mobilization achieved through VLA4 targeting. This revealed VLA4 as a promising target associated with mobilization of a clinically relevant magnitude. Compared with mobilization with G-CSF, long-term serially repopulating HSCs were relatively more strongly enriched in VLA4 inhibitor plus CXCR2 agonist–mobilized grafts than clonogenic cells without long-term repopulating capacity, indicating preferential targeting of a very immature HSPC fraction. Given how brisk the mobilization occurred and assuming BM sinusoids as the exit route for HSCs, our findings support the notion of a close proximity of the mobilization-sensitive HSCs to BM sinusoids. Moreover, the numbers of rapidly mobilizable HSCs markedly exceeded previous estimates. Furthermore, we found an unexpected contribution of stromal (endothelial) CXCR2 to the mobilization induced by tGro-β alone as well as by VLA4 antagonist plus tGro-β, hinting at a previously unsuspected crosstalk between granulocytes and stroma as a master regulator of HSPC localization and egress.

The cytokine G-CSF and chemotherapy-triggered myeloid rebound, the 2 commonly utilized clinical mobilization regimens, are comprised of 2 partly overlapping steps, proliferation and the actual release of the HSPCs from the BM (53, 54). Although not necessary nor sufficient for mobilization per se, proliferation (and therefore expansion) of HSPCs clearly contributes to the magnitude of G-CSF and chemotherapy-induced HSPC mobilization (55). In addition to the obvious increase in numbers, HSPC localization has been reported to change over the course of proliferation, favoring closer proximity to the vessels, which in turn facilitates BM exit (56, 57). In comparison, the rapid kinetics of mobilization with VLA4 antagonist plus tGro-β (peak reached within 15–30 minutes of s.c. injection) would not allow for cell division to occur prior to the HSPC release from the BM. Therefore, the relative enrichment for primitive, serially repopulating units in the VLA4 antagonist plus tGro-β–mobilized blood grafts likely reflects their preferential targeting and a preferential vascular (sinusoidal) localization. Compared with G-CSF and chemotherapy, much less severe alterations in the BM composition in the course of VLA4 antagonist plus tGro-β–mediated mobilization are to be expected, which is particularly important in light of the very similar potency.

Mature hematopoietic cells contained in the transplant have been shown to directly influence HSPC engraftment. For example, the presence of regulatory T cells was demonstrated to ensure HSPC survival and colonization of the host BM (58). Also, bystander effects of the myeloid compartment, granulocytes in particular, for the establishment of the niche following irradiation/transplantation were recently described (59). Thus, granulocyte-derived mastat. However, mobilization was stronger in B6 MMP9-KO mice than in their WT counterparts. Conversely, tGro-β- and AMD3100 plus tGro-β–induced mobilization was significantly reduced in FVB MMP9-KO mice or following MMP inhibitor treatment, but it was not altered in B6 MMP9-KO mice. Interestingly, the combination of CWHM-823 plus tGro-β mobilized with equal efficiency in both knockout strains relative to WT control mice, whereas mobilization was slightly, but not significantly, reduced in mice pretreated with batimastat. Taken together, these data do not support a clear role of MMP9 in CWHM-823 plus tGro-β–associated augmented mobilization. Rather, they suggest differences in the mechanism underlying mobilization induced by combining CXCR2 stimulation and VLA4 blockade compared with CXCR2 activation in conjunction with CXCR4 blockade.
TNF-α was shown to facilitate vasculogenesis, a critical step during regeneration. Given the high percentage as well as the direct stimulation of neutrophils in VLA4 antagonist plus tGro-β-mobilized (and also G-CSF-mobilized) blood specimens, it is conceivable that granulocyte-derived factors contribute to the superior performance of the corresponding grafts. While not excluding this possibility, the results of our LSK gene expression profiling further imply inherent differences in the profile and composition of the differentially mobilized versus BM resident HSPC populations themselves. In line with the idea that differences within the HSPC fraction (i.e., a higher proportion of more immature, serially repopulating cells) are primarily responsible for the observed graft fitness, LSK SLAM cells purified from AMD3100 plus tGro-β-mobilized blood (a regimen very similar to ours) were found to outperform those isolated from G-CSF–treated blood donors when both were tested head-to-head in a competitive transplant assay against BM cells (52).

Neutrophils have been demonstrated to be indispensable for IL-8–induced (and G-CSF–induced) mobilization (51, 60). Therefore, our observation of a significant reduction of tGro-β as well as CWHM-823 plus tGro-β–associated mobilization in the context of granulocyte deficiency was in line with expectations. By contrast, the subsequent findings that nonhematopoietic CXCR2-expressing cells coregulate HSPC egress triggered by tGro-β alone and that the presence of the receptor in either one of the compartments, stroma or hematopoietic cells, was in fact sufficient to elicit HSPC egress when the combination of CWHM-823 plus tGro-β was used, were very much surprising. Thus, while the presence of granulocytes was necessary for both, tGro-β and CWHM-823 plus tGro-β–induced mobilization, lack of CXCR2 expression on their surface seemed, to a large extent, to be compensated for in the case of CWHM-823 treatment, as long as the stromal CXCR2 was present.

Relevant for the interactions between HSPC and neutrophils, a role of CXCR2 expression in endothelia has been increasingly recognized. Accordingly, CXCR2 expression in ECs of the lung vessels and its involvement in controlling neutrophil passage from the circulation into the lung parenchyma have been described (38). CXCR2 signaling has also been shown to critically mediate EC proliferation and therefore vascular repair after transplantation (39). Moreover, involvement of the zebrafish CXCR2 ortholog CXCR1 in the hematopoietic stem cell niche remodeling during embryogenesis has been demonstrated (61). Our findings along with the results of a companion study (unpublished observations) imply an additional, immediate role of endothelial CXCR2 in the regulation of HSPC retention versus egress.

Significant controversy surrounds the role of matrix metalloproteases in general and MMP9 in particular for mobilization regimens involving neutrophil stimulation such as tGro-β, IL-8, or...
G-CSF. In a recent study by Hoggatt et al. describing a mobilization approach similar to ours (AMD3100 plus tGro-β, ref. 52) the authors reported a contribution of MMP9 activity to the synergistic mobilization response in mice. Moreover, a correlation between mobilization efficiency in healthy G-CSF donors and activity of the MMP9 inhibitor TIMP1 was described. In our analysis, a trend toward a more pronounced contribution of MMP9 during HSPC egress induced by AMD3100 plus tGro-β as compared with CWHM-823 plus tGro-β was observed. However, a requirement for intact MMP9 for a tGro-β-based regimen could not be corroborated.

The differences in the magnitude and kinetics of HSPC egress associated with the different regimens analyzed in this study, as well as the distinct properties of cells mobilized with VLA4 antagonists alone, versus tGro-β alone, versus VLA4 antagonist plus tGro-β, can be interpreted as a result of targeting distinct HSPC subsets (Figure 6A–D). Thus, based on their performance in the competitive transplantation setting (Figure 2, A–C), more mature progenitor cells are primarily mobilized with a VLA4 antagonist alone (Figure 6B). Expression of the beta 2 integrins LFA1 and Mac1 on human and murine hematopoietic progenitor cells (HPCs) but not stem cells has been demonstrated (48, 62). Hence, although not markedly dislodging stem cells when blocked individually, inhibition of LFA1 or Mac1 in conjunction with VLA4 blockade resulted in enhanced HPC mobilization. Consistent with the direct mode of action of VLA4 antagonists (4, 63), loss of granulocytes did not affect VLA4 inhibitor–induced HSPC egress.

Peak mobilization after tGro-β treatment is remarkably fast. Activation of CXCR2 signaling in ECs has been reported to lead to EC contraction (64, 65) and weakening of intercellular junctions (66, 67). In accordance with these reports, we detected increased permeability of vascular cells after CXCR2 stimulation in vivo and in vitro (unpublished observations). Increased vascular leakiness has also been found in the BM of tGro-β-treated mice (52) and is likely a major contributor to the brisk kinetics of tGro-β–triggered HSPC emigration (Figure 6C). Moreover, though not addressed in our analysis directly, an involvement of the complement cascade, a key orchestrator of pharmacologically and pathologically induced HSPC release from the BM (68, 69), and the associated release of anaphylatoxins might have facilitated increased vascular permeability. The latter are further known to stimulate the release of the lipolytic enzyme phospholipase C β2 (PLCB2), which in turn disrupts the lipid raft formation needed for optimal signaling via VLA4 and CXCR4 (68, 70).

Adhesive interactions and cross-stimulation between CXCR2–expressing granulocytes and endothelia are critical for the increased permeability and likely also for the subsequent cell-cell contact or soluble mediator-enabled HSPC release in the context of mobilization with tGro-β alone. However, in contrast to the reported requirement of LFA1 for IL-8–induced mobilization (19), neither deficiency for LFA1 nor for Mac1 enhanced tGro-β–mediated HSPC egress. Loss of one beta 2 integrin was possibly compensated for by the presence of the other, along with other integrins. With regard to the HSPC population targeted, the transplant data suggested predominant mobilization of progenitor cells with tGro-β, as was the case with VLA4 antagonist alone. Hence, elevated vascular permeability and reduced retention induced by tGro-β alone are not sufficient to release the HSCs from their protective BM environment.

Finally, when tGro-β is combined with a second stimulus in the form of VLA4 inhibition, in addition to the reciprocal stimulation of CXCR2-expressing cells, the associated changes in permeability and cell contact and/or soluble mediator decreased HSPC retention, which is further hit by lack of VLA4 signaling (Figure 6D). As demonstrated by the results of the serial competitive transplantation analysis, this combination of events indeed recruited with high efficiency highly engraftable, true HSCs into the circulation. Cross-stimulation of tGro-β–targeted cells that were dependent on integrin interactions, and blockade of both VLA4 and either LFA1 or Mac1 appears to disturb rolling and adhesion and therefore proximity of CXCR2–expressing cells to an extent that cannot be compensated, unlike when tGro-β is given alone. This concept could explain the relatively less efficient mobilization with VLA4 antagonist plus tGro-β in mice lacking beta 2 integrins.

In summary, we show that simultaneous administration of CXCR2 agonists in conjunction with VLA4 small-molecule inhibitors results in rapid and robust mobilization of HPCs and HSCs. A contribution of hematopoietic (neutrophils) and nonhematopoietic (endothelial) CXCR2 to the mobilization response was found. Grafts mobilized with the VLA4 inhibitor plus tGro-β combination exhibited serial repopulating capacity. Given several advantages of this combination over currently approved HSPC mobilization methods, it represents an exciting potential strategy for clinical development in the future. We are continuing to optimize the pharmacokinetic and pharmacodynamic properties of the VLA4 inhibitors to enable future large-animal efficacies and toxicology studies in nonhuman primates, which are currently underway. Further, our data support the proposed perisinusoidal localization of very primitive (true) HSCs and will also serve as a tool to learn more about the interplay between mature hematopoietic cells, nonhematopoietic stroma, and stem cells.

Methods

Mice

Male 6- to 12-week-old WT BALB/c (CD45.2+), BALB/cByJ (CD45.2+), and syngeneic CByJ.SJL(B6)-Ptprca/J (CD45.1+) as well as WT C57BL/6J and syngeneic B6.SJL-Ptprc+ Pep3+/BoyJ (CD45.1+), WT DBA/2J, and FVB/NJ mice were purchased from the Jackson Laboratory. The knockout strains C.129S2(B6)-Cxxr2tm1Mwm/J (CXCR2-KO, BALB/cj background), B6.FVB(Cg)-Mmp9tm1Tvu/J (MMP9-KO, C57BL/6J background), FVB.Cg-Mmp9tm1Tvu/J (MMP9-KO, FVB/NJ background), B6.129S7-Igaltm1Bll/J (CD11a-KO, C57BL/6 background), and B6.129S4--Igamt1Myd/J (CD11b-KO, C57BL/6 background) were also obtained from Jackson Laboratory. Endothelial-specified CXCR2 ablated mice were generated by crossing C57BL/6-Cxxr2tm1Rmra/J (CXCR2fl/fl, Jackson Laboratory) and B6;129-Tg (Cdhs5-cre)1Spe/J (Cdhs5Cre, Jackson Laboratory) mice. Mobilization experiments were performed with Cxcr2fl/wtCre+ mice (CXCR2fl/wtCre+ were used as controls) reconstituted with WT BM from syngeneic B6.SJL-Ptprc+ Pep3+/BoyJ (CD45.1+, Jackson Laboratory) donors. Following lethal irradiation (1× 9.5–11 Gy, using a cesium source) and transplantation, mice were kept on 0.5 mg/ml sulfamethoxazole and 0.1 mg/ml trimethoprim (HI-Techn Pharmacal), administered by mouth in drinking water.
Peripheral blood (PB) and BM cells were recovered as previously described (12). Cell counts (WBC) were measured on an automatic hemocytometer (Hemavet 950, Drew Scientific). Total counts (per tissue) as well as the majority of functional analysis were performed with filtered and otherwise unmanipulated cell suspensions, whereas red cells were removed from suspensions prepared for flow cytometry using hypotonic lysis. Absolute numbers of immunophenotypically defined populations (e.g., LSK cells) were calculated assuming CD45<sup>+</sup> cells are equivalent to WBC from the corresponding cell count analysis.

In vivo reagents and treatments

Human recombinant truncated Gro-β peptide (tGro-β, CXCL2, SB-251353 from GlaxoSmithKline) was used for all in vivo experiments. Stock solution stored at –80°C was freshly thawed and diluted immediately prior to the s.c. injection (2.5 mg/kg) (52). Murine recombinant CXCL1 (PeproTech) and human recombinant CXCL8 (aa 23-99, Sino Biological) stock solutions were prepared in water. All chemokines were diluted in PBS (GE Healthcare Life Sciences) or used directly in the CWHM-823 solution. CWHM-823 plus chemokine mixtures were sonicated in a water bath for 15–30 minutes prior to injection to counteract precipitation. Firategrast was synthesized based on the published structure (C<sub>27</sub>H<sub>27</sub>F<sub>2</sub>NO<sub>6</sub>; patent no. US2014051655), dissolved in a 1% ethanol solution in PBS and injected i.v. or s.c. (100 mg/kg). Mice treated with firategrast plus tGro-β received 2 separate injections. All other VLA4 antagonists, BIO5192 (TOCRIS, Bio-Techne Corporation) (28), CWHM-842, -822, -823, -824, and -825 were dissolved in DMSO (100× stock solution) and diluted in NaHCO<sub>3</sub>/NaCl buffer (1:1, 10 mM NaHCO<sub>3</sub>, pH 8; 0.9% wt/vol NaCl) for subsequent i.v., s.c. (CWHM-823 only), or i.p. (CWHM-823 only) injection at 3 mg/kg. BIO5192 plus tGro-β and CWHM-842 plus tGro-β treatment consisted of 2 separate injections, whereas CWHM-823 plus Gro-β was administered either as 2 injections (VLA4 antagonist i.v., tGro-β s.c.) or as one injection (i.v., i.p., or s.c.) as specified for each experiment. AMD3100 (Mozobil, Genzyme) suspension was prepared in PBS and administered s.c. (5 mg/kg). AMD3100 plus tGro-β mixture was prepared by diluting tGro-β in the AMD3100 solution and then s.c. injected. RhG-CSF (Neupogen, Filgrastim, Amgen) diluted in PBS was injected i.p. every 12 hours at a dose of 100 μg/kg for a total of 4 (day 3) or 9 (day 5) doses. Broad spectrum matrix metalloproteinase inhibitor batimastat (BB-94, APExBio) was dissolved in DMSO, diluted in corn oil (Sigma-Aldrich), vortexed thoroughly, and injected i.p. 12 and 2 hours (25 mg/kg) prior to the mobilization treatment. Control mice received DMSO/corn oil injections. In all other experiments, mice injected with PBS, PBS/DMSO, or left untreated (baseline) at the time point of analysis are referred to as control animals throughout the manuscript.

Depletion of granulocytes

To deplete granulocytes in vivo the anti-mouse Ly6G/Ly6C antibody (Gr1, clone RB6-8C5, BioXCell) was administered i.v. (200 μg/mouse) 36 hours prior to the mobilization treatment. Control mice were treated with an isotype control antibody (clone 2A3, BioXCell). Efficient depletion of peripheral blood granulocytes was confirmed using differential blood count as well as flow cytometric analysis.
Colony-forming unit assay
Cells were incubated in duplicate in commercially available growth factor supplemented with methylcellulose medium for mouse CFU-Cs (Stem Cell Technologies or R&D Systems) as described (10, 12). CFU-Cs (BFU-E, CFU-GM, and CFU-GEMM) were enumerated after 6-8 days of culture.

Quantitative real-time PCR
For analysis of gene expression in cell populations (LSK, LSK SLAM, and different mature cell fractions) sorted from the BM RNA was isolated using RNA XS column kit (Macherey-Nagel). Ambion Turbo DNA-free Kit (Thermo Fisher Scientific) was then used to remove genomic DNA followed by reverse transcription of the RNA using the QuantiTect Reverse Transcription Kit (Qiagen). qRT-PCR (genomic DNA followed by reverse transcription of the RNA using the DNA-free Kit (Thermo Fisher Scientific) was then used to remove isolated using RNA XS column kit (Macherey-Nagel). Ambion Turbo and different mature cell fractions) sorted from the BM RNA was isolated using RNA XS column kit (Macherey-Nagel). RNA from LSK cells sorted from untreated BM or PB of different mature cell fractions was then used to remove 1/x-weighted linear regression. The potency of compounds in blocking ligand binding to integrins was determined by modification of our previously described methods (71). Briefly, purified human VCAM1/Fc chimera protein (R&D Systems) was diluted to 1 g/ml in TBS +/0.1% BSA blocking buffer (TBS + with 1% bovine serum albumin). Recombinant human integrin ITGA4/ITGB1 (α4β1; VLA4) or ITGA4/ITGB7 (α4β7) (R&D Systems) was diluted to 1 μg/ml in TBS’/0.1% bovine serum albumin. Test compounds were diluted into the integrin solution and added to the washed ligand-coated plate according to a standard template with each sample repeated in triplicate. After incubation for 2 hours at room temperature, the plate was washed 3 times with 150 μl TBS’-buffer. To each well, biotinylated anti-β1 (catalog BAF1778, R&D Systems) or anti-α4 antibody (clone 7.2R, R&D Systems) was added at 1:100 dilution. Control samples were stained with PE-labeled donkey anti-human IgG (catalog 507-116-098, Jackson Immunoresearch Laboratories) added at 1:100 dilution. Control samples were stained with PE-labeled donkey anti-human IgG (catalog 017-110-006, Jackson Immunoresearch Laboratories, 1:100 dilution). Secondary antibody staining was performed at RT for 30 minutes. Following addition of 7-AAD for 5 minutes at RT, cells were washed twice with HBSS/BSA buffer and analyzed by flow cytometry.

Water solubility assay
For each VLA4 antagonist tested, 1-2 mg solid compound was placed in an Eppendorf tube with 1 ml equilibrium solubility buffer (ESB, 50 mM citric acid, 50 mM K2HPO4, 50 mM Ammonium, and 50 mM KCl, pH 7) and incubated in a Thermomixer for 48 hours at 25°C at 500 rpm. After 24 hours, tubes were centrifuged at 3000g for 5 minutes, and an aliquot of the supernatant was removed and diluted into linear standard range of the standard curve for the LCMS method. Sample dilutions were made in 75:25 ESB/acetomitrile. Firategраст, BIO5192, CWHM-823, CWHM-824, CWHM-825, and CWHM-842 concentrations were determined on a Sciex API-4000 LC/MS system (SCIEX) in positive electrospray mode. Analytes were eluted from an Armor C18 reverse phase column (2.1 x 30 mm, 5 μm) using a 0.1% formic acid mobile phase system with aqueous to acetonitrile gradient over 3.7 minutes at a flow rate of 0.35 ml/min. Peak areas for the mass transition of m/z 500.3 > 454.2 for firategраст, m/z 817.2 > 394.0 for BIO5192, m/z 505.0 > 176.0 for CWHM-822, m/z 518.0 > 472.2 for CWHM-823, m/z 621.2 > 575.0 for CWHM-824, m/z 747.6 > 192.2 for CWHM-825, and m/z 523.4 > 390.2 for CWHM-842 were integrated using Analyst 1.5.1 software (SCIEX). Peak areas were plotted against standard concentrations with a 1/x-weighted linear regression.

Microarray analysis
RNA from LSK cells sorted from untreated BM or PB of differentially mobilized (G-CSF [5 days], AMD3100, or CWHM-823 plus Gro-β; single injection) mice was prepared using the RNA XS column kit (Macherey-Nagel) and hybridized to the Mouse Gene Expression Panel 12). CFU-Cs (BFU-E, CFU-GM, and CFU-GEMM) were enumerated using Analyst 1.5.1 software. Peak areas were plotted against standard concentrations with a 1/x-weighted linear regression.

Pharmacokinetics
Thus, log 2 scale-transformed kit (Macherey-Nagel) and hybridized to the Mouse Gene Expression Panel (Applied Biosystems) listed in Supplemental Table 7.

Pharmacokinetics
Cells were incubated in duplicate in commercially available growth factor supplemented with methylcellulose medium for mouse CFU-Cs (Stem Cell Technologies or R&D Systems) as described (10, 12). CFU-Cs (BFU-E, CFU-GM, and CFU-GEMM) were enumerated after 6-8 days of culture.

Pharmacokinetics
For analysis of gene expression in cell populations (LSK, LSK SLAM, and different mature cell fractions) sorted from the BM RNA was isolated using RNA XS column kit (Macherey-Nagel). Ambion Turbo DNA-free Kit (Thermo Fisher Scientific) was then used to remove genomic DNA followed by reverse transcription of the RNA using the QuantiTect Reverse Transcription Kit (Qiagen). qRT-PCR (genomic DNA followed by reverse transcription of the RNA using the DNA-free Kit (Thermo Fisher Scientific) was then used to remove isolated using RNA XS column kit (Macherey-Nagel). Ambion Turbo and different mature cell fractions) sorted from the BM RNA was isolated using RNA XS column kit (Macherey-Nagel). RNA from LSK cells sorted from untreated BM or PB of different mature cell fractions was then used to remove 1/x-weighted linear regression. The potency of compounds in blocking ligand binding to integrins was determined by modification of our previously described methods (71). Briefly, purified human VCAM1 (R&D Systems) was diluted to 5 μg/ml in TBS’ buffer (25 mM Tris pH 7.4, 137 mM NaCl, 2.7 mM KCl, 1mM CaCl2, 1 mM MgCl2, 1 mM MnCl2) was added to wells of a 96-well transparent microtiter plate and incubated overnight at 4°C. Wells were washed 3 times with ‘TBS’ and blocking buffer (TBS’ with 1% bovine serum albumin), the plate was incubated for 1 hour at 37°C, and then washed 3 times with TBS’ buffer. Recombinant human integrin ITGA4/ITGB1 (α4β1; VLA4) or ITGA4/ITGB7 (α4β7) (R&D Systems) was diluted to 1 μg/ml in TBS’/0.1% bovine serum albumin. Test compounds were diluted into the integrin solution and added to the washed ligand-coated plate according to a standard template with each sample repeated in triplicate. After incubation for 2 hours at room temperature, the plate was washed 3 times with 150 μl TBS’-buffer. To each well, biotinylated anti-β1 (catalog BAF1778, R&D Systems) or anti-α4 antibody (clone 7.2R, R&D Systems) at 1 μg/ml in TBS’/0.1% BSA

Pharmacokinetics
The potency of compounds in blocking ligand binding to integrins α4β1 and α4β7 was determined by modification of our previously described methods (71). Briefly, purified human VCAM1 (R&D Systems) was diluted to 5 μg/ml in TBS’ buffer (25 mM Tris pH 7.4, 137 mM NaCl, 2.7 mM KCl, 1mM CaCl2, 1 mM MgCl2, 1 mM MnCl2) was added to wells of a 96-well transparent microtiter plate and incubated overnight at 4°C. Wells were washed 3 times with ‘TBS’ and blocking buffer (TBS’ with 1% bovine serum albumin), the plate was incubated for 1 hour at 37°C, and then washed 3 times with TBS’ buffer. Recombinant human integrin ITGA4/ITGB1 (α4β1; VLA4) or ITGA4/ITGB7 (α4β7) (R&D Systems) was diluted to 1 μg/ml in TBS’/0.1% bovine serum albumin. Test compounds were diluted into the integrin solution and added to the washed ligand-coated plate according to a standard template with each sample repeated in triplicate. After incubation for 2 hours at room temperature, the plate was washed 3 times with 150 μl TBS’-buffer. To each well, biotinylated anti-β1 (catalog BAF1778, R&D Systems) or anti-α4 antibody (clone 7.2R, R&D Systems) at 1 μg/ml in TBS’/0.1% BSA
was added and the plate covered and incubated for 1 hour at RT. After washing the plate 3 times with TBS buffer, streptavidin-conjugated horseradish peroxidase (R&D Systems) diluted in TBS blocking buffer was added to the wells and the plate incubated for 20 minutes at room temperature. The plate was washed 3 times with TBS buffer followed by addition of 50 μl TMB substrate (MilliporeSigma). After incubation for 20 minutes at RT, plates were read by colorimetric detection at 650 nm wavelength using a Tecan Safire II plate reader. Concentration-response curves were constructed by nonlinear regression (best fit) analysis, and IC50 values were calculated for each compound.

To evaluate the broader selectivity of test compounds, potency against a nontargeted beta-1 subunit–containing integrin, ITGα2β1, was measured as previously described (71). Assessment of compound potency against 2 selected members of the RGD-binding integrin family, ITGA5/ITGB1 (α5β1) and ITGAV/ITGB3 (αvβ3), was determined by a similar method in which binding of the purified human integrins (R&D Systems) was assessed to plates coated with their respective purified ligands, human fibronectin (2 μg/ml, R&D Systems) and human vitronectin (1 μg/ml; R&D Systems). Biotinylated anti-ITGAV (αv) or anti-ITG5 (α5; R&D Systems) antibodies were used to detect the stably bound integrins in conjunction with streptavidin-conjugated horseradish peroxidase as described above. The αvβ3 and α5β1 assays were validated by inclusion of a known potent inhibitor of these integrins, CWHM-12 (71), while the α2β1 assay was validated by inclusion of another previously described inhibitor, compound 8 (data not shown) (72).

Specificity screening
tGm-β1 was sent out to DiscoverX (DiscoverX Corporation) for blinded profiling against the gpcrMAX panel (148 G protein–coupled receptors screened in both, agonist and antagonist mode) using the PathHunter beta-arrestin enzyme fragmentation (EFC) technology.

Statistics
Data are mean ± SEM unless indicated otherwise. Linear mixed models were used to analyze experiments with data repeatedly measured from the same mice, while analysis of variance (ANOVA) was used for data from independent samples. A logarithm transformation was performed as necessary to better satisfy the normality and homoscedasticity assumptions (see Figure legends for details). Ad-hoc multiple comparisons were also used for between-group differences of interest. The resultant P values were adjusted by Holm’s step-down Bonferroni’s adjustment. Compared with the widely used Bonferroni’s adjustment, a step-down method is more powerful (smaller adjusted P values) while maintaining strong control of the familywise error rate. All analyses were 2-sided and significance was set at a P value of 0.05. The statistical analyses were performed using SAS 9.4 (SAS Institutes).

Study approval
All animals were housed at the Washington University Medical School vivarium under SPF conditions with autoclaved chow and water ad libitum. All experiments were performed in accordance with the guidelines of the Washington University Animal Studies Committee, approved by the IACUC in agreement with AAALAC guidelines.

Author contributions
DK, MPR, and JFD conceived of the study. DK, MPR, JR, SC, LG, EC, DC, MOE, MH, JZ, GA, HC, EW, WY, LGE, RFH, SDA, MJM, MJP, DWG, PGR, and DMM performed experiments. DK, MPR, and DC analyzed data. WY assisted with microarray data processing and analysis. FG assisted with the statistical analysis of the data. DK, MPR, AT, HBB, DCL, and JFD supervised research. DK wrote the manuscript. All authors discussed, commented on, and approved the final version of the manuscript.

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Address correspondence to: John F. DiPersio, Division of Oncology, Washington University School of Medicine, 660 South Euclid Avenue, Campus Box 8007, St. Louis, Missouri 63110, USA. Phone: 314.454.8491; Email: jdipersi@wustl.edu.

RFH and SDA’s present address is: Confluence Discovery Technologies, St. Louis, Missouri, USA.

MJM’s present address is: Department of Chemistry, Saint Louis University, St. Louis, Missouri, USA.

MJP’s present address is: HTS Robotics Core, Washington University School of Medicine, St. Louis, Missouri, USA.

DWG’s present address is: Department of Molecular Microbiology & Immunology, Saint Louis University School of Medicine, St. Louis, Missouri, USA.


