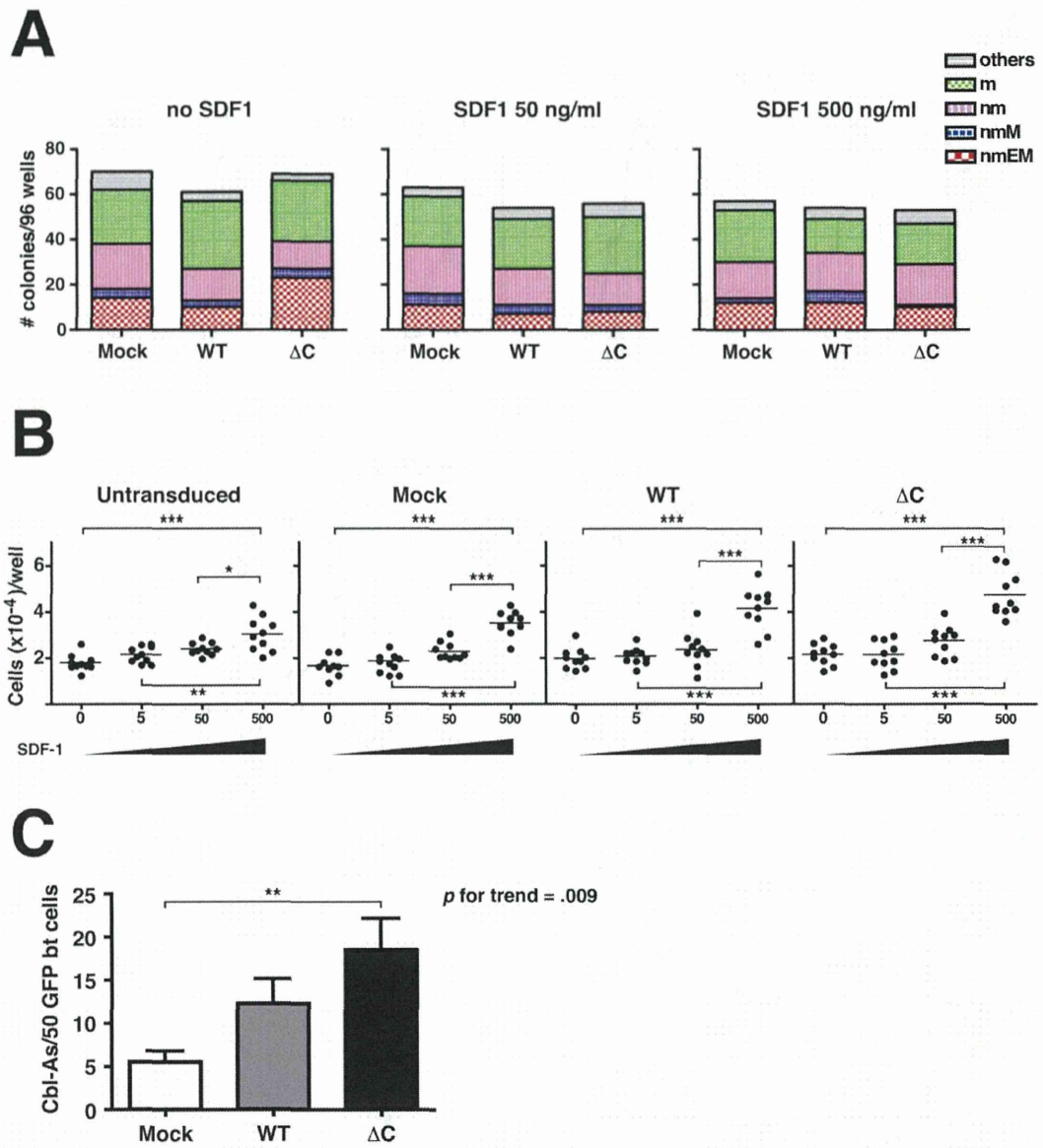


Figure 2. Augmentation of C-X-C chemokine receptor type 4 (Cxcr4) signaling in hematopoietic stem/progenitor cells (HS(P)Cs) correlates with enhanced proliferative responses in the presence of SDF-1 in high concentrations and of feeder cell layers. **(A):** Single-cell cultures in the presence of SCF, TPO, IL-3, and EPO as baseline cocktail. Cultures were maintained either with no other additives (no SDF-1) or added exogenous SDF-1 at the indicated concentrations (SDF-1 50 or 500 ng/ml). Shown are colony numbers (numbers of positive wells in a 96-well plate) and the colony types assessed on day 11. Cell composition morphologically determined in each colony was represented by a single or combination of the following letters; m: macrophage, n: neutrophil, E: Erythroblast, M: Megakaryocyte. For example, "m" means a colony containing only macrophages, whereas "nm" and "nmM" represent colonies composed of a mixture of corresponding cell lineages. "nmEM" represents colonies derived from "uncommitted"single cells with high potential for multilineage differentiation within a myeloid compartment. **(B):** Proliferation ability of *Cxcr4*-modified cells in response to SDF-1. Test HS(P)Cs were sorted at 50 cells per well into 96-well plates and were counted 7 days after cultivation in serum-free basal medium (containing SCF and TPO) alone or in the presence of SDF-1. Data shown represent three independent experiments ($n = 10$). Mean values are indicated as bars. *, $p < .05$; **, $p < .01$; ***, $p < .001$. Untransduced: Untransduced control. **(C):** Ability of *Cxcr4*-modified HS(P)Cs to form Cbl-As in the presence of feeder cells. Test cells were directly sorted onto a feeder layer of C3HT101/2 cells at 50 cells per well. Numbers of areas per well evaluated on day 10 are shown as mean values ± SD ($n = 4$, representative of three independent experiments). **, $p < .01$. Abbreviations: Cbl-As, cobblestone-like areas; GFP, green fluorescent protein; SDF-1, stromal cell-derived factor 1; WT, wild type.

from previous observations [14, 16–18, 39, 40], was clearly observed in transduced 32D cells with 100 ng/ml of SDF-1 (Supporting Information Fig. S5A). With this low concentration of SDF-1, however, as the degree of receptor internalization in both Mock- and WT-Cxcr4-transduced cells was far less in HS(P)Cs than that observed in other cells, including the

human T cell line CEM (Supporting Information Fig. S5C), blunting of the response of ΔC-Cxcr4 was therefore not evident (Supporting Information Fig. S5B). Nevertheless, HS(P)Cs overexpressing ΔC-Cxcr4 showed a blurred response in receptor internalization with a higher concentration of SDF-1 (500 ng/ml, Fig. 1B). Gain-of-function effects were confirmed in WT- and ΔC-Cxcr4-transduced cells in comparison with Mock-cells; a stepwise enhancement in transwell migration to SDF-1/CXCL12 was evident with greater response for ΔC-Cxcr4-cells (Fig. 1C; Supporting Information Fig. S6), consistent with an enhanced chemotactic response in human cells expressing WHIM-type CXCR4 [17, 18, 30, 41–43]. The stepwise nature of gain-of-function for WT- and ΔC-Cxcr4 was further confirmed with a Ca^{2+} influx assay using 32D cells (Supporting Information Fig. S7).

Enhanced In Vitro Proliferative Response of HS(P)Cs Under Particular Circumstances by Gain-of-Function Modification in Cxcr4 Signaling

We next examined the characteristics of these cultured HS(P)Cs using a series of in vitro assays. At first, clonogenic ability and differentiation characteristics were compared in in vitro single-cell liquid culture. As shown in Figure 2A, modification of Cxcr4 did not alter frequencies of HS(P)Cs capable of colony formation, with colony types also unaffected (no SDF-1). Addition of SDF-1 produced no remarkable influence on these cultures (SDF-1 50 and 500 ng/ml). These results suggest minimal influences of Cxcr4 receptor modification on HS(P)Cs' clonogenic ability in stroma-free liquid culture and on their differentiation properties. We next tested proliferative characteristics of bulk populations in liquid culture. As described (Supporting Information), our basal culture system is distinct from others in allowing self-renewal of murine HSCs both at a single cell level [24, 44] and at a population level [45] with the minimum cytokines (SCF and TPO) needed in a serum-free setting. In this so-called "HSC-self-renewal compatible" culture, we found fresh HSCs highly resistant to desensitization to Sdf-1 (Supporting Information Fig. S8). When cultured HS(P)Cs were tested in this system (input cells = 50 cells per well), massive expansion was observed for all cell types in the absence of SDF-1/CXCL12 (Fig. 2B, SDF-1: 0 ng/ml). Cell proliferation was unexpectedly enhanced in the presence of SDF-1/CXCL12 especially at the highest concentration (500 ng/ml), which was considered "desensitizing" for most cell types [16, 46, 47]. Interestingly, the additive effects of exogenous Cxcr4 receptors on HS(P)C proliferation became evident only in the presence of 500 ng/ml SDF-1; as shown in Supporting Information Figure S9, stepwise (i.e., WT < ΔC-Cxcr4) enhancement in response was observed in comparison with control samples (Untransduced and Mock).

We then tested whether overexpression of exogenous Cxcr4 affected HSPCs' ability to colonize and to repopulate C3H10T1/2 feeder cell layers in an in vitro coculture assay [33]. In this assay, test cells can be scored for in vitro clonogenic ability in the presence of stromal cells by counting characteristic cobblestone-like areas that form underneath the feeder layers. We first confirmed both the presence of Sdf-1 in this culture supernatant and the significance of the Sdf-1/Cxcr4 axis for cobblestone-like area formation using the CXCR4 antagonist AMD3100 (Supporting Information Fig. S10). When Cxcr4-modified HS(P)Cs were subjected to this assay, the ability to form cobblestone-like areas proved remarkably enhanced in comparison with that in Mock control cells; enhancement occurred in a stepwise manner, with the greatest response in ΔC-Cxcr4-overexpressing cells (Fig. 2C). Collectively, these findings demonstrated that augmented Cxcr4 signaling alters cellular responses to favor HSPC survival/proliferation, especially in the presence of high concentrations of Sdf-1 and/or of supporting feeder layers that produce this ligand.

Augmented Cxcr4 Signaling in Murine HS(P)Cs/HSPCs Does not Enhance BM Homing/Lodging but Improves Subsequent BM Repopulation

We then examined how augmentation in Cxcr4 signaling affected the in vivo behavior of transplanted HS(P)Cs at different times during BM reconstitution. As the earliest process, BM homing efficiency was assessed using $EGFP^+$ cultured HSPC populations (Supporting Information Figs. S1B, S2A). As shown, we found no enhancement in BM homing of HSPCs at the indicated times (4–24 hours) by gain-of-function Cxcr4 modification (Fig. 3A; Supporting Information Fig. S2B). This was also true when we used $EGFP^+$ cultured HS(P)Cs as the sole transplants and assessed BM homing by counting number of CFCs recovered from each recipient at 16 and 24 hours (Fig. 3B). We next examined subsequent BM repopulation kinetics by extending the times of analysis, similarly as previously reported [33]: in this setting, CFCs measurable in BM are supposed to reflect not only homing but also subsequent cell division shortly after homing. As shown in Figure 3C, augmentation of Cxcr4 signaling in HS(P)Cs did not lead to increased numbers of CFCs in recipient BM at days 2 and 4. Collectively, these results demonstrate that augmentation in murine Cxcr4 signaling does not affect the earliest events after HSPC transplantation, including BM homing/lodging by HSPCs.

We further tracked in vivo BM repopulation by donor HS(P)Cs beyond the above time points. As recipients of HS(P)Cs alone barely survived lethal-dose irradiation beyond day 7, test HS(P)Cs were transplanted with competitor cells, with their relative contributions to BM repopulation determined at days 14 and 21 (Fig. 3D). Of note is that stepwise increases in donor chimerism were visible in BM at these time points (p for trend = .038) with ΔC-Cxcr4-cells showing the highest donor cell contribution (*vs.* Mock, $p = 0.035$).

The advantage in BM repopulation for Cxcr4-augmented HS(P)Cs was also noticeable in cohorts of long-term recipients (Fig. 3E, p for trend = .042), again with ΔC effects being the highest (vs. Mock, $p = .038$). From these observations, we conclude that the Sdf-1/Cxcr4 axis plays a role in productive BM repopulation by transplanted HSPCs after the second phase (weeks 2–3) that follows transplantation.

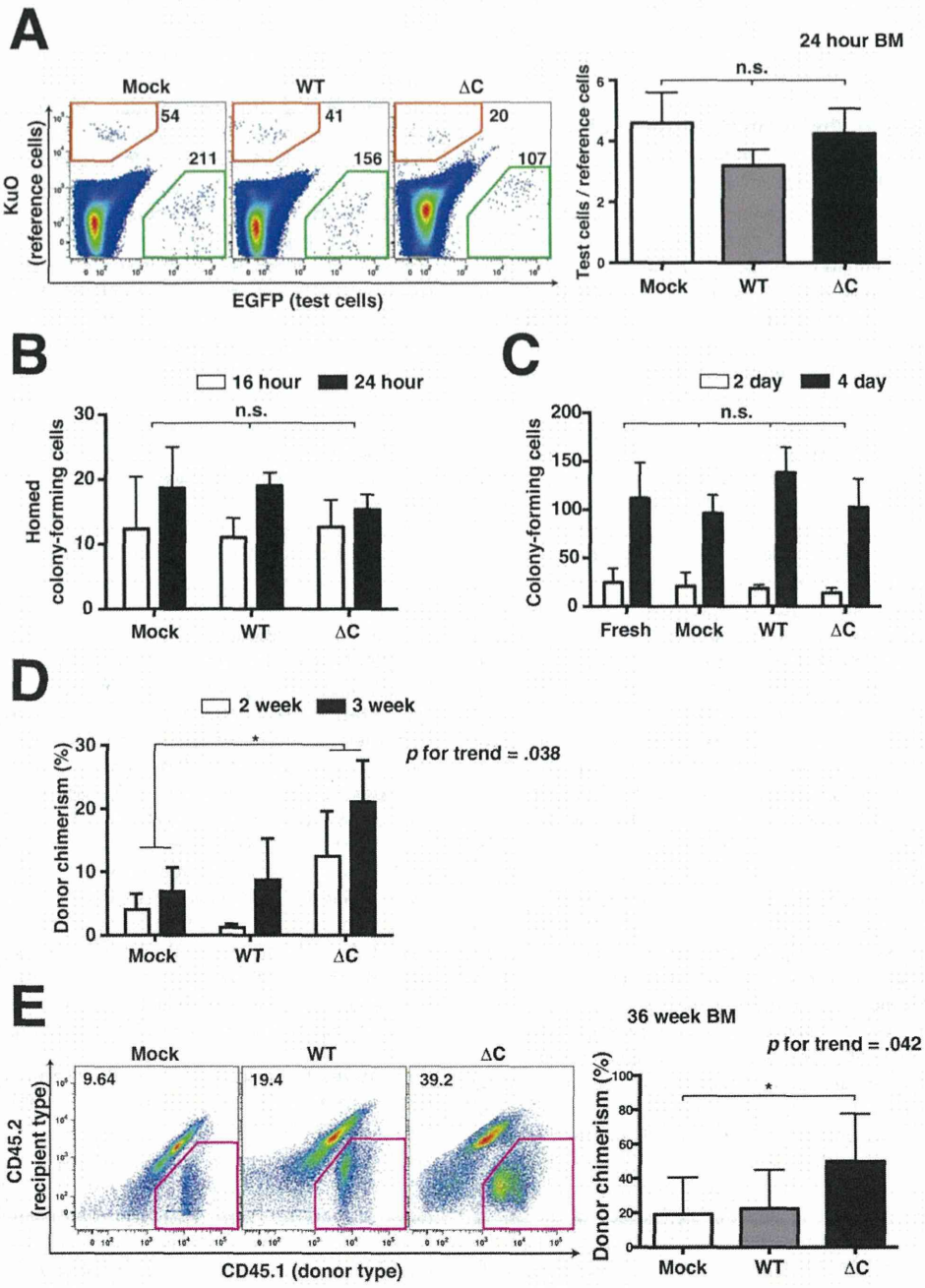
Continuous Overexpression of Exogenous Cxcr4 Receptors in HS(P)Cs Leads to Poor Peripheral Reconstitution

The possible correlation between Cxcr4 signal intensity in HS(P)Cs and their short-term BM reconstitution efficiency made us wonder how long-term transplantation outcome would fare in gain-of-function experiments. In competitive repopulation assays, ΔC-Cxcr4-transduced HS(P)Cs contributed poorly to PB chimerism at 4 weeks (data not shown) and 16

weeks (Fig. 4A), consistent with the major features of WHIM syndrome, lymphocytopenia and neutropenia [15, 16, 19, 41, 48]. That overexpression of WT-Cxcr4 in HS(P)Cs also did not improve PB reconstitution (Fig. 4A, WT) was unexpected, as beneficial effects of similar treatment are reported for human $CD34^+$ cells [13, 14]. Systemic injection of the Cxcr4 antagonist AMD3100 into long-term recipients increased PB donor chimerism significantly in WT- and ΔC-Cxcr4 groups, suggesting that blunted peripheral mobilization of donor cells played a causal role in poor PB reconstitution in these mice (Fig. 4B; Supporting Information Fig. S11).

Enhanced Donor Cell Chimerism Occurs in BM Cells Throughout Developmental Stages of HS(P)Cs Expressing Gain-of-Function Cxcr4 Receptors

Of note is that the recipients of ΔC-Cxcr4-transduced HS(P)Cs showed obvious long-term donor cell chimerism in BM (Fig. 3E); this may accompany favorable in vivo expansion of



transplanted HSCs or may simply indicate accumulation of certain cell types at a particular differentiation stage, thus reflecting conditions that in some respect are abnormal. To distinguish these possibilities, we scrutinized recipient BM for donor cell chimerism in multiple cell compartments, including the long-term HSC fraction (LT-HSC; Supporting Information Fig. S12). As shown in Figure 4C, individual recipients of Mock-treated HS(P)Cs did not display significant alterations in donor cell chimerism among these populations, suggesting little influence on hematopoiesis by Mock transduction of HS(P)Cs. In contrast, donor cell chimerism differed clearly between PB leukocytes (open histograms) and BM compartments (colored histograms) in each recipient that received Cxcr4 signal-enhanced HS(P)Cs (Fig. 4C, WT and ΔC). General enhancement of chimerism in the BM compartments, including LT-HSC, was striking, with no difference between fractions, which suggested that Cxcr4 signal-dependent donor cell expansion might occur even at a stem cell level. When donor cell chimerism was similarly assessed along two major paths of lineage development, that is, B cells and neutrophils, results were consistent with the idea that Cxcr4 signal augmentation induced no gross alterations, such as maturation arrest, in hematopoietic development, but likely caused exaggerated BM retention of donor cells (Fig. 4C). The increase of "phenotypically-defined" LT-HSCs might not necessarily imply, however, that functional HSC numbers were amplified. We could demonstrate persistence within primary recipient BM of reconstituting cells that were capable of establishing donor chimerism upon serial transplantation (Supporting Information Fig. S13, PB). No advantage over Mock-treated cells, however, was observed for WT- and ΔC-Cxcr4-expressing cells in repopulation of secondary recipient BM (Supporting Information Fig. S13, BM), unlike the case with primary recipients (Fig. 3E) (see Discussion section).

Loss-of-Function Studies Support the Proposed Roles of Cxcr4 in Murine HSPCs in Transplantation

To understand Cxcr4's roles in murine HSPCs better in transplantation, we conducted loss-of-function analyses in experimental settings like those used for the gain-of-function studies. To this end, we mostly used purified HSCs obtained from *Cxcr4* conditional KO mice [49] and littermate (LM) control mice after induction (Supporting Information). First, the absence of Cxcr4 receptors did not affect HSCs' colony-forming ability in liquid medium or their multilineage potentials (Fig. 5A). When cell proliferation was tested in liquid culture, we found loss of SDF-1-responsiveness in KO-HSCs (Fig. 5B, KO) whereas control LM-HSCs showed a response similar to that observed in both fresh B6 HSCs (Supporting Information Figs. S8, S15A) and cultured HS(P)Cs (Fig. 2B). Consistent with the loss of SDF-1-response phenotypes, the ability of Cxcr4-deficient HSCs to form cobblestone-like areas was shown to be severely impaired (Fig. 5C). The defect in Cxcr4-KO-HSCs found in this assay may be regarded as a mirror image of the results in gain-of-function experiments (Fig. 2C), thus further supporting the importance of Cxcr4 receptors in HSC/HSPC colonization and proliferation in the presence of feeder cell environments that produce Sdf-1/Cxcl12.

We then examined how the absence of Cxcr4 signaling in HSCs affected in vivo kinetics of donor cell repopulation. Early BM homing was assessed using fresh HSPCs with or without receptor desensitization using the Cxcr4 antagonist AMD3100 following established preincubation methods [37–39]. As assessed at 15 hours, we did not detect a decrease in BM homing of HSPCs that had been rendered unresponsive to Sdf-1/Cxcl12, even with additional systemic administration of AMD3100 (Fig. 5D; Supporting Information Fig. S3). Supporting this observation, assessment of subsequent repopulation in BM on days 4 and 6 demonstrated that in the absence of Cxcr4 expression (KO), transplanted HSCs yielded numbers of CFCs comparable with those observed for wild type HSCs (LM; Fig. 5E). Genotyping polymerase chain reaction demonstrated that most randomly picked-up CFCs had the Cxcr4 locus completely knocked out (31 in 33, 93.9%), thus indicating that the "highly purified HSCs" that we injected alone into lethally irradiated recipients were capable of BM homing in vivo and colony formation ex vivo in the absence of functional Cxcr4 expression. KO-HSCs, contrary to these findings, clearly showed significant impairment in capability of BM repopulation at 2 and 3 weeks after transplantation (Fig. 5F). As expected, KO-HSCs contributed to PB long-term less than did LM control HSCs (Fig. 5G), consistent with previous observations [7, 8, 12].

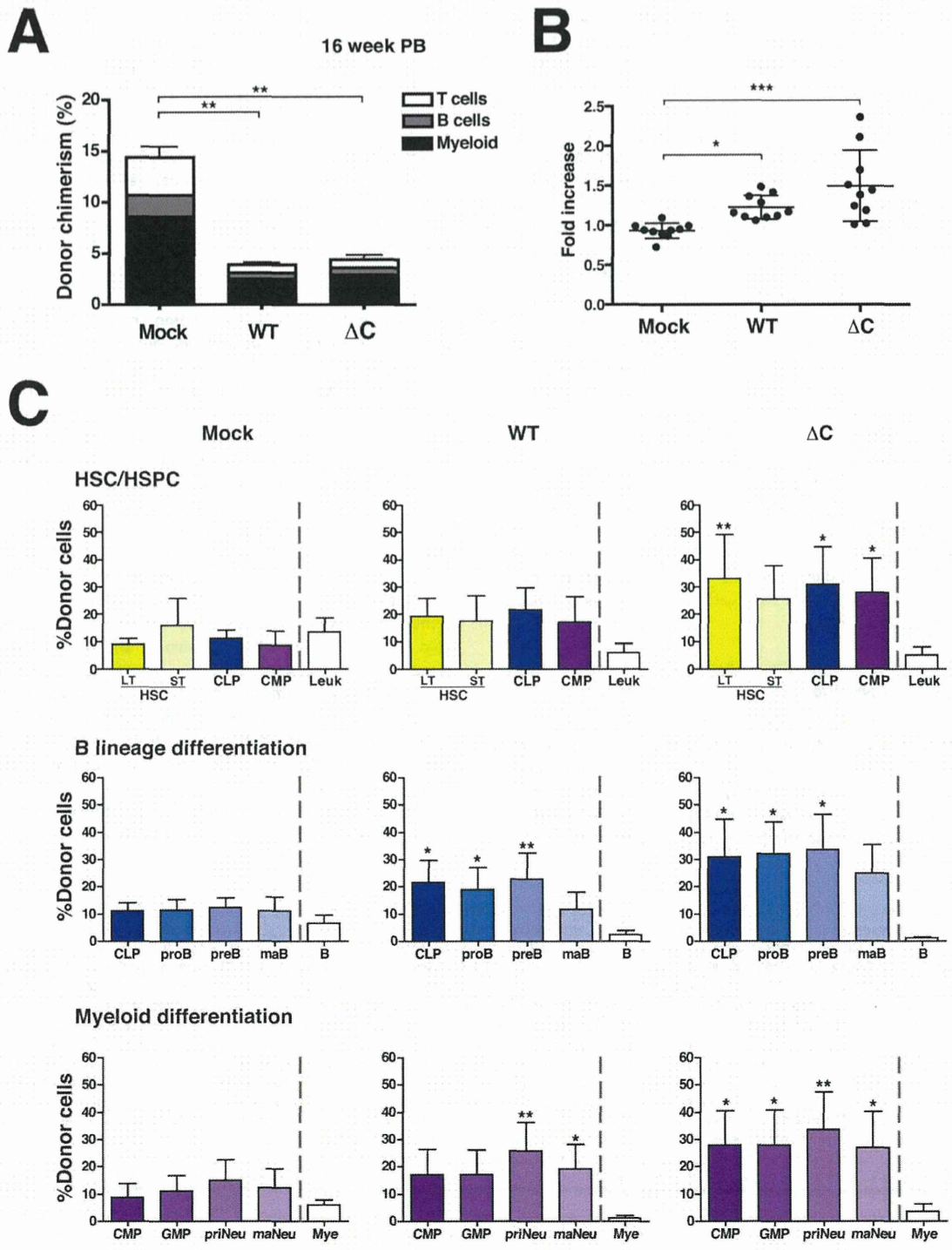
Our comprehensive approach has pointed out the importance of the Sdf-1/Cxcr4 axis in HSC/HSPCs for productive

Figure 3. Augmentation of C-X-C chemokine receptor type 4 (Cxcr4) signaling does not affect early homing/lodging processes but likely enhances subsequent BM repopulation by murine hematopoietic stem/progenitor cells (HS(P)Cs). Tracking in vivo fates within recipient BM of reconstituting HS(P)Cs with or without overexpression of gain-of-function Cxcr4 receptors. **(A):** Homing assay using cultured HSPCs, either Mock-virus-treated (Mock) or expressing either WT or C-terminal truncated-type (ΔC) exogenous Cxcr4 receptors. These test cells (50,000 cells, $EGFP^+$) and fresh c-$Kit^+Sca\text{-}1^+$Lineage marker-negative cells expressing KuO protein (17,000 cells) were intravenously infused into lethally irradiated recipient mice. Twenty-four hours later, homing events were quantified in recipient BM as KuO^+ and $EGFP^+$ cells (red and green gates, respectively). Representative flow cytometry analysis is shown at left. Shown are the event ratios of $EGFP^+$ (test cells) to KuO^+ (reference cells) expressed as means ± SD ($n = 5$ for each group). **(B, C):** Assessment of BM homing/early repopulation processes. Shown are numbers of colony-forming cells detected in BM of each mouse that received either fresh unmodified HSCs or HS(P)Cs (200 cells per mouse) alone after lethal-dose irradiation. Shown are the results obtained at 16 hours (open histograms) and 24 hours (closed histograms) after transplantation **(B)**, or at 2 days (open histograms) and 4 days (closed histograms) after transplantation **(C)**. Data are expressed as means ± SD ($n = 4$, representative of three independent experiments). **(D):** Donor cell chimerism assessment in competitive repopulation assays. Shown are donor cell chimerism values in recipient BM at week 2 (open histograms) and week 3 (closed histograms). Mean values ± SD are shown ($n = 4$, representative of four independent experiments). *, $p < .05$ (ΔC vs. Mock). Values of p for trend are shown. **(E):** Donor cell chimerism in long-term recipient BM at week 36. Shown is representative flow cytometry analysis of donor cell chimerism in mice transplanted with treated HS(P)Cs in a competitive repopulation assay. Graphic representation of chimerism analysis is also shown as mean values ± SD ($n = 5$, representative of three independent experiments). *, $p < .05$ (ΔC vs. Mock). Values of p for trend are shown. Abbreviations: BM, bone marrow; ΔC, C-terminal deletion; EGFP, enhanced green fluorescent protein; KuO, Kusabira Orange; WT, wild type.

engraftment and repopulation in recipient BM, especially for a particular phase after transplantation, that is, the early/sub-early repopulation phase (~2–3 weeks), which follows the initial engraftment process (<1 week).

Alteration in Phosphorylation Kinetics of Erk in Response to SDF-1 in HS(P)Cs Expressing ΔC-Cxcr4

To obtain mechanistic insights, we examined how overexpression of exogenous Cxcr4 receptors altered downstream



signaling events in HS(P)Cs. Phosphorylation kinetics in Erk1/2 and Akt were tested in $EGFP^+$ cultured HS(P)Cs by flow cytometry analysis. While phosphorylated Akt intensity was unaltered before and after stimulation (Supporting Information Fig. S15), clear alterations in phosphorylated Erk1/2 (pErk) signals confirmed that the HS(P)Cs used the MAPK/Erk signaling pathway in response to SDF-1/CXCL12 (Fig. 6). The peak response was seen 3 minutes after stimulation, with the greatest response in ΔC-Cxcr4-expressing cells. Response termination was delayed in ΔC-Cxcr4-expressing cells, with residual pErk-signals still detectable at 15–30 minutes, whereas cells in the other two samples (Mock and WT) quickly returned to baseline status by 15 minutes. Of note is that with even a "desensitizing-high" concentration of SDF-1 (500 ng/ml), ΔC-expressing cells still showed visibly enhanced phosphorylation at peak and delay in its termination. Similar behavior was confirmed using the 32D murine hematopoietic cell line (Supporting Information Fig. S4D).

Discussion

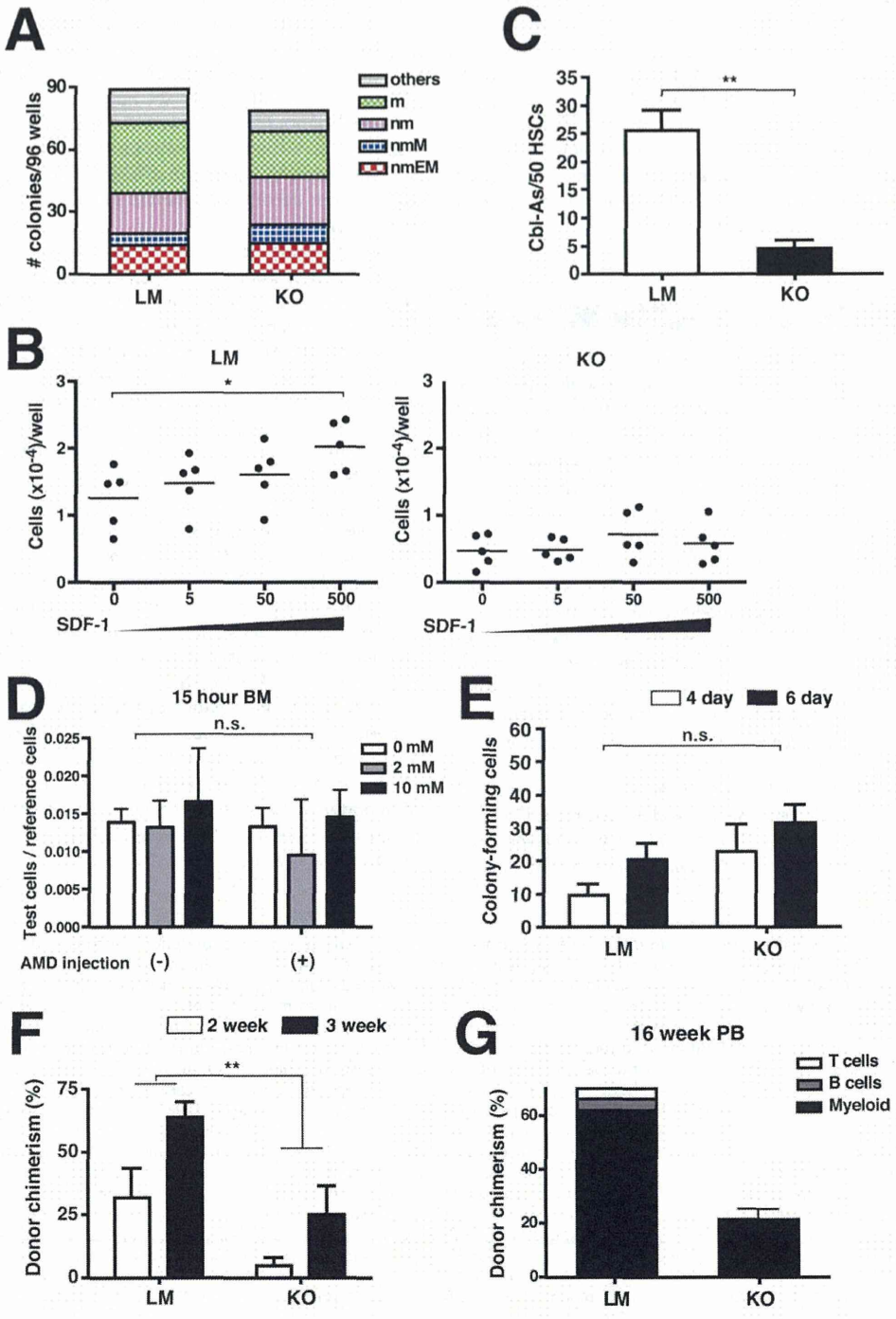
Our goal is to use experimental findings to benefit patients undergoing transplantation. To this end, we sought to clarify the stage-specific role of Cxcr4 signaling in transplanted cells during hematopoietic reconstitution. With gain-of-function experiments, augmentation of Cxcr4 signaling appeared relatively unimportant in accelerating the homing/lodging of murine HSC/HSPCs but efficient in enhancing their subsequent repopulation of BM. This pattern of stage-specificity in Cxcr4's role was also demonstrated in a series of loss-of-function experiments. Our results strengthen the generally held idea that CXCR4 signal modification can benefit transplantation outcomes [13, 14], by demonstrating enhancement in donor cell expansion, possibly even at a stem cell level. Also to note, however, is that sustainment of augmented signal led to detrimental effects on transplantation outcomes in this study, even with overexpression of a non-mutant Cxcr4 receptor.

That the SDF-1/CXCR4 axis plays an indispensable role in BM homing by adult HSCs seems widely accepted [50, 51]; this may contrast with our findings. This view, however, rests on pioneering studies that used human HSPCs, mostly in loss-of-function settings [12, 52]. These were followed by gain-of-function studies that demonstrated improved human HSPC engraftment in immunodeficient mice with overexpression of wild-type CXCR4 [13, 14, 19]. KO mouse studies have clearly demonstrated the importance of Cxcr4 for robust hematopoietic reconstitution in transplantation but have not specifically addressed BM homing by purified HSCs [7, 8]. Our study of the role of Cxcr4 is, so far as we know, the first gain-of-function approach to use murine HSC/HSPCs. Using cultured HSPCs derived from KSL cells, we demonstrated that overexpression of exogenous Cxcr4 receptors did not lead to enhanced BM homing (Fig. 3A; Supporting Information Fig. S2). Short-term homing (16–24 hours) and subsequent repopulation kinetics (days 2–4) in BM, assessed by using cultured HS(P)Cs, also did not alter with Cxcr4 overexpression (Fig. 3B, 3C). These results indicate that augmentation of Cxcr4 signaling does not benefit transplanted murine HSC/HSPCs in these early processes.

In loss-of-function experiments, we used purified HSCs freshly isolated from Cxcr4-deficient mice for the assessment of early BM repopulation capability (days 4 and 6; Fig. 5E). The results supported the idea that the Sdf-1/Cxcr4 axis may be of little importance in this phase of BM repopulation. The impact of Cxcr4 signaling on early BM homing was assessed using fresh, uncultured HSPCs following established desensitizing methods using the Cxcr4 antagonist AMD3100 [37–39]. Our results demonstrated that BM homing (15 hours) of purified HSPCs did not much depend on the Sdf-1/Cxcr4 axis, consistent with previous observations [37]. There may be controversy, however, on this issue, because other workers observed impaired BM homing by HSPCs after AMD3100 treatment [38, 39]. This can probably be attributed to differences in study design, with the most significant one being that we used HSPCs alone as the sole transplant/analyte whereas others assessed homing of HSPCs by injecting donor cells preincubated with AMD3100 as either low-density BM cells [38] or lineage-negative BM cells [39]. We emphasize the importance of these observations, as in clinical settings unpurified hematopoietic cells are most often used in transplantation. Nevertheless, we believe that our findings also are of importance, because clinical gene therapy trials currently in progress use purified $CD34^+$ HSPCs [53], thus resembling our experimental settings.

Figure 4. Continuous expression of gain-of-function receptors in HS(P)Cs leads to impaired peripheral reconstitution, but enhanced bone marrow (BM) repopulation, by donor cells throughout developmental stages. **(A):** Percent PB chimerism of donor cells on competitive repopulation assay 16 weeks after transplantation. Donor cell chimerism was separately determined for each lineage (T cells, B cells, and myeloid cells). Shown are data represented as mean values ± SD obtained from mice each of which received 700 enhanced green fluorescent protein positive cells along with 2×10^5 competitor cells ($n = 5$, representative of three independent experiments). **, $p < .01$. **(B):** Assessment of AMD3100-induced mobilization effect on donor chimerism in long-term recipients. One week after the analysis of baseline donor cell chimerism, each mouse received intraperitoneal systemic injection of AMD3100 (10 mg/kg). One hour after AMD3100 injection, PB chimerism was analyzed and the fold increase of donor chimerism was calculated for each donor. Shown are data represented as mean values ± SD obtained from groups of recipient mice ($n = 10$, representative of two independent experiments). *, $p < .05$; ***, $p < .001$. Individual data are shown in Supporting Information Fig. S11. **(C):** Donor cell chimerism assessed 36 weeks after transplantation in long-term recipient BM (colored histograms) and PB (white histograms) for multiple hematopoietic subfractions. Top: Stem cells, progenitor cells, and PB leucocytes (HSC/HSPC). Middle: Differentiation path along B cell development (B lineage differentiation). Bottom: Differentiation path along myeloid development (myeloid differentiation). Detailed marker combinations used to define each population are listed (Supporting Information Materials). Data analyzed by Dunnett's multiple comparison test with peripheral chimerism as control. *, $p < .05$; **, $p < .01$. Abbreviations: B, B cells in PB; ΔC, C-terminal deletion; CLP, common lymphoid progenitors; CMP, common myeloid progenitors, all in BM; GMP, granulocyte/macrophage progenitors; HSPC, hematopoietic stem/progenitor cell; Leuk: leukocytes in PB; LT-HSC, long-term hematopoietic stem cells; maB, mature B cells; maNeu, mature neutrophils, all in BM; Mye, myeloid cells in PB; PB, peripheral blood; proB, pro-B cells; preB, pre-B cells; all in BM; priNeu: primitive neutrophils; ST-HSC, short-term hematopoietic stem cells; WT, wild type.

In contrast to the above findings, studies of donor cell reconstitution within BM at weeks 2 and 3 clearly accorded a role to Cxcr4 signaling (Figs. 3D, 5F). Although the frequency of donor HSCs in BM could not be determined by immunophenotyping at these early time points, the results obtained by cobblestone-like area formation experiments (Fig. 2C) mirrored in vivo donor chimerism at 2–3 weeks (Fig. 3D). This in turn supports the idea that augmentation of Cxcr4 signaling leads to expansion (colonization, proliferation) of HSPCs in vivo, where the presence of stromal cells is expected to promote this event.



Cxcr4-mediated effects on in vivo HSC/HSPC expansion may arise from improved survival/proliferation and/or enhanced retention of HSPCs within BM. Consistent with this idea, studies suggested that CXCR4 relays a survival-promoting signal within HSPCs [51, 54]. Of note is that Cxcr4 signal-mediated enhancement in proliferation was more evident when HSPCs either were in contact with feeder cells (Fig. 2C) or were stimulated with SDF-1 in high concentrations (500 ng/ml, Fig. 2B; Supporting Information Fig. S9). Promotion of cell cycling in HSPCs by SDF-1/CXCL12 may conflict with recent descriptions of a role for Cxcr4 in maintenance of HSC quiescence [7, 8, 11]. We, however, think it possible to explain this discrepancy by the difference in culture systems. In fact, in our defined culture system HSCs, either fresh or after stimulation, resisted desensitization to high concentrations of SDF-1 (Figs. 2B, 5B; Supporting Information Fig. S8) [44, 45]. As we could readily detect measurable levels of Sdf-1 in murine BM cavities irrespective of irradiation conditioning (Supporting Information Fig. S10B), one can expect local levels of Sdf-1 to be very high in vivo, especially in the functional niche environment. We therefore speculate that in our scenario, high concentrations of Sdf-1 may favor proliferative response in murine HSC/HSPCs when they exist in "self-renewal-compatible" conditions. Altered Erk activation kinetics may be responsible for the observed enhancement of repopulation capability in HSC/HSPCs equipped with gain-of-function Cxcr4, but further investigation is needed for more mechanistic insights.

When considering implications of our study for clinical transplantation, poor PB contribution by HS(P)Cs overexpressing WT-Cxcr4 is significant; this may contrast with the favorable effects of CXCR4 overexpression on the engraftment/repopulation of human HSPCs [13, 14]. Species differences may account for this discrepancy. Differences in study design, however, may contribute more importantly. We transplanted only cells expressing EGFP at high levels (thus likely exogenous Cxcr4 receptors as well, see Supporting Information Fig. S4A), which might have led to unfavorable, extreme gain-of-function effects on hematopoietic reconstitution. Alternatively, improved reconstitution reported in the case of CXCR4 overexpression may be attributable to the coinjection of non-HSC/HSPCs, so-called "facilitating cells" [50], mostly missing in our study.

We must note that as our work required the cultivation of HSCs in gain-of-function settings, thus our findings may be applicable to cycling HSC/HPSCs (likely present in the setting of gene therapy) but not to their fresh counterparts. Our culture system, however, is capable of maintenance of progenitor phenotypes in most cells after 7 days (Supporting Information Fig. S14B, S14C) and actually is "self-renewal compatible" [44, 45]. We therefore believe that this study complements general knowledge concerning the role of CXCR4 in HSPC transplantation [51, 55, 56].

Kawai et al. [19] demonstrated that transplantation of human HSPCs overexpressing WHIM-mutant receptor resulted in the WHIM phenotype in the mouse environment. More recently, generation of WHIM-type mice was reported with many interesting findings [16]. Ours is the first study of the WHIM-type receptor in the murine system in the context of HSPC transplantation. Most notable is that enhanced BM repopulation was observed in the LT-HSC population, at least as defined by immunophenotyping, upon signal augmentation (Fig. 4C). Secondary transplantation, however, did not confirm stem cell expansion in primary BM by definition (Supporting Information Fig. S13, PB); this was partly explained by the properties of "defects in PB release," which should inhere in phenotypically defined HSCs still expressing exogenous Cxcr4. Furthermore, the highest donor chimerism by ΔC-Cxcr4-expressing cells in BM (Fig. 3E) was not phenocopied to the BM of secondary recipients (Supporting Information Fig. S13, BM), probably suggesting impaired repopulating ability in each donor LT-HSC due to long-term nonphysiological Cxcr4 signaling events. Further investigation is necessary for formal demonstration of whether enhanced HSC self-renewal is

Figure 5. Loss-of-function studies support the importance of the Sdf-1/Cxcr4 axis in HSC/hematopoietic stem/precursor cell (HSPCs) for BM repopulation in the early/sub-early phase after transplantation. All loss-of-function studies (except those illustrated in (D)) used as starting materials purified HSCs harvested from either *Cxcr4* conditional KO mice or their LM control mice similarly treated with pIpC. **(A):** Single-cell cultures in the presence of SCF, TPO, IL-3, and EPO. Shown are colony numbers and the colony types assessed on day 14. m: macrophage, n: neutrophil, E: Erythroblast, M: Megakaryocyte (classifications, m, nm, nmM, and nmEM). **(B):** Proliferation ability of either LM HSCs or *Cxcr4*-KO HSCs in response to SDF-1 (starting from 50 cells per well). Data shown represent three independent experiments ($n = 5$). Mean values are indicated as bars. *, $p < .05$. **(C):** Ability of either LM HSCs or *Cxcr4*-KO HSCs to form Cbl-As in a feeder layer of C3H10T1/2 cells (50 input cells per well). Shown are numbers of areas per well evaluated on day 10 as mean values ± SD ($n = 4$, representative of three independent experiments). **, $p < .01$. **(D–F):** Tracking in vivo fates of reconstituting HSC/HSPCs in the initial (**D**, < 24 hours) and early/sub-early (**E**, days 4–6; F, weeks 2–3) phases within BM. **(D):** BM homing assay using fresh HSPCs. Test cells were from Kusabira Orange transgenic (KuO-Tg) mice (10,000 KuO^+ c-$Kit^+Sca\text{-}1^+$Lineage marker-negative (KSL) cells). Fresh BM cells from EGFP-Tg mice were used as reference cells (10^6 cells). Test cells either were left untreated or were incubated with AMD3100 for 30 minutes at 37°C at the indicated concentration before transplantation. Lethally irradiated recipients received a mixture of test and reference cells. Fifteen hours later, homing events were quantified in recipient BM as KuO^+ and $EGFP^+$ cells. One group of mice received PBS intraperitoneally [AMD injection (−)], whereas another received AMD3100 [AMD injection (+)]. Shown are the event ratios of test cells (KuO^+) to reference cells (enhanced green fluorescent protein positive) as means ± SD (n = 4 for each group). **(E):** Shown are numbers of colony forming cells detected in BM of each mouse that received either LM HSCs or KO HSCs (100 cells per mouse) at day 4 (open histograms) and day 6 (closed histograms). Data are expressed as means ± SD ($n = 5$, representative of two independent experiments). **(F):** Donor cell chimerism in recipient BM at week 2 (open histograms) and week 3 (closed histograms) is shown as mean values ± SD ($n = 5$, representative of three independent experiments). **, $p < .01$. **(G):** Percent PB chimerism of donor cells on competitive repopulation assay 16 weeks after transplantation. Donor cell chimerism was separately determined for each lineage (T cells, B cells, and myeloid cells). Shown are data represented as mean values ± SD obtained from mice each of which received 100 $CD34^-KSL$ cells along with 2×10^5 competitor cells. Abbreviations: BM, bone marrow; Cbl-As, cobblestone-like areas; HSC, hematopoietic stem cell; KO, knock out; m, colony containing only macrophages; nm and nmM, colonies composed of a mixture of corresponding cell lineages; nmEM, colonies derived from "uncommitted"single cells with high potential for multilineage differentiation within a myeloid compartment; LM, litter mate; PB, peripheral blood; SDF-1, stromal cell-derived factor 1.

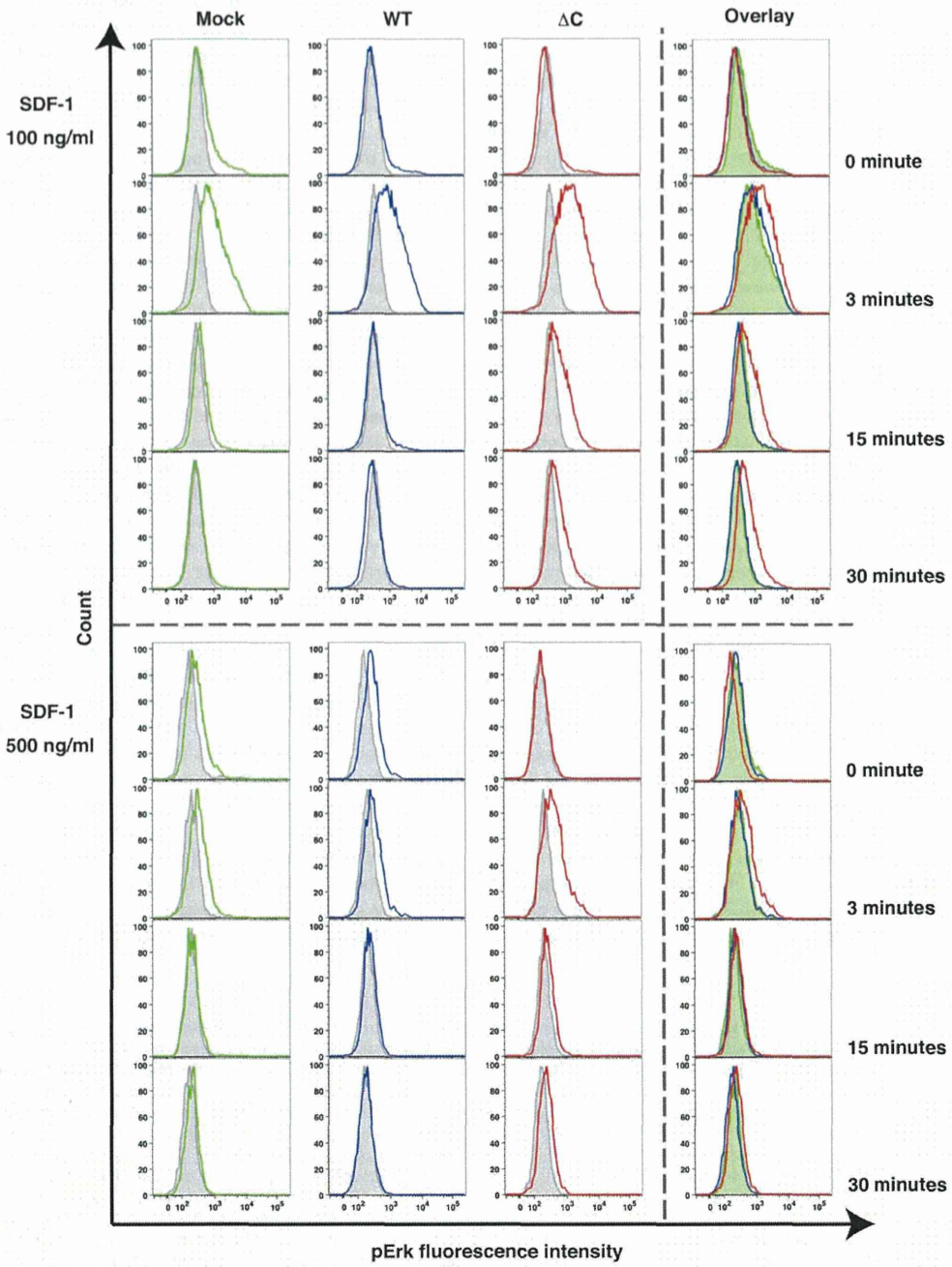


Figure 6. Augmentation of C-X-C chemokine receptor type 4 signaling leads to enhanced and prolonged phosphorylation of Erk in hematopoietic stem/progenitor cells upon SDF-1 stimulation. Altered Erk activation kinetics. Test cells were cytokine-starved and stimulated with 100 or 500 ng/ml SDF-1 for the indicated times. The amount of phosphorylated Erk was determined by flow cytometry analysis. Top, each colored histogram represents pErk intensity at different time points upon stimulation with 100 ng/ml SDF-1. Bottom, same as top panel except for SDF-1 concentration (500 ng/ml). Gray histogram in each represents isotype-control. Overlay histogram figures are shown on the right for comparison between groups. Abbreviations: pErk, phospho extracellular signal-regulated kinase; SDF-1, stromal cell-derived factor 1; WT, wild type.

feasible, using an experimental system that allows in vivo inducible expression of exogenous Cxcr4 in test cells for a certain period of time after transplantation. More practically, it would be intriguing, for clinical application of our findings, to aim at drug discovery by screening for an efficacious and specific small molecule having characteristics of a CXCR4 agonist with limited desensitization. With elucidation of mechanisms underlying CXCR4/Cxcr4 signal-mediated effects, perhaps combined multiple strategic approaches will eventually culminate in greater clinical benefits.

Conclusion

We have addressed stage-specific roles for Cxcr4 signaling in donor cell repopulation in BM for the first time using purified mouse HSC/HSPCs. With unique combinational approaches that used both loss-of-function and gain-of-function modification of Cxcr4 receptors, we found that Cxcr4 signaling appears unimportant for the homing/lodging of mouse HSC/HSPCs but vital for their subsequent repopulation of BM. Cxcr4 signal enhancement likely favored BM repopulation by donor cells at a level of primitive cell populations, but was shown to be detrimental to PB reconstitution when sustained too long. Consequently, we think it important to investigate further when and how long signaling via this chemokine receptor is to be modified in order to favorably enhance HSPC engraftment in future transplantation medicine.

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Author Contributions

C.-Y.L. and M.O.: conception and design, collection and/or assembly of data, data analysis and interpretation, and manuscript writing; S.Y., M.O., S.S., and S.K.: collection and/or assembly of data; Y.M., Y.I., M.O., and Y.I.: provision of study material or patients; M.N.: data analysis and interpretation; H.N.: financial support and manuscript writing.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflict of interest.

References

1 Buckley RH. Transplantation of hematopoietic stem cells in human severe combined immunodeficiency: Longterm outcomes. Immunol Res 2011;49:25–43.

2 Copelan EA. Hematopoietic stem-cell transplantation. N Engl J Med 2006;354: 1813–1826.

3 Jenq RR, van den Brink MR. Allogeneic haematopoietic stem cell transplantation: Individualized stem cell and immune therapy of cancer. Nat Rev Cancer 2010;10: 213–221.

4 Cancelas JA, Jansen M, Williams DA. The role of chemokine activation of Rac GTPases in hematopoietic stem cell marrow homing, retention, and peripheral mobilization. Exp Hematol 2006;34:976–985.

5 Wilson A, Oser GM, Jaworski M et al. Dormant and self-renewing hematopoietic stem cells and their niches. Ann N Y Acad Sci 2007;1106:64–75.

6 Nagasawa T, Omatsu Y, Sugiyama T. Control of hematopoietic stem cells by the bone marrow stromal niche: The role of reticular cells. Trends Immunol 2011;32:315–320.

7 Nie Y, Han YC, Zou YR. CXCR4 is required for the quiescence of primitive hematopoietic cells. J Exp Med 2008;205:777–783.

8 Sugiyama T, Kohara H, Noda M et al. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. Immunity 2006;25:977–988.

9 Wright DE, Bowman EP, Wagers AJ et al. Hematopoietic stem cells are uniquely selective in their migratory response to chemokines. J Exp Med 2002;195:1145–1154.

10 Kawabata K, Ujikawa M, Egawa T et al. A cell-autonomous requirement for CXCR4 in long-term lymphoid and myeloid reconstitution. Proc Natl Acad Sci USA 1999;96:5663–5667.

11 Tzeng YS, Li H, Kang YL et al. Loss of Cxcl12/Sdf-1 in adult mice decreases the quiescent state of hematopoietic stem/progenitor cells and alters the pattern of hematopoietic regeneration after myelosuppression. Blood 2011;117:429–439.

12 Peled A, Petit I, Kollet O et al. Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science 1999;283:845–848.

13 Brenner S, Whiting-Theobald N, Kawai T et al. CXCR4-transgene expression significantly improves marrow engraftment of cultured hematopoietic stem cells. Stem Cells 2004;22:1128–1133.

14 Kahn J, Byk T, Jansson-Sjostrand L et al. Overexpression of CXCR4 on human CD34+ progenitors increases their proliferation, migration, and NOD/SCID repopulation. Blood 2004;103: 2942–2949.

15 Hernandez PA, Gorlin RJ, Lukens JN et al. Mutations in the chemokine receptor gene CXCR4 are associated with WHIM syndrome, a combined immunodeficiency disease. Nat Genet 2003;34:70–74.

16 Balabanian K, Brotin E, Biajoux V et al. Proper desensitization of CXCR4 is required for lymphocyte development and peripheral compartmentalization in mice. Blood 2012; 119:5722–5730.

17 Diaz GA, Gulino AV. WHIM syndrome: A defect in CXCR4 signaling. Curr Allergy Asthma Rep 2005;5:350–355.

18 McDermott DH, Lopez J, Deng F et al. AMD3100 is a potent antagonist at CXCR4(R334X), a hyperfunctional mutant chemokine receptor and cause of WHIM syndrome. J Cell Mol Med 2011;15:2071–2081.

19 Kawai T, Choi U, Cardwell L et al. WHIM syndrome myelokathexis reproduced in the NOD/SCID mouse xenotransplant model engrafted with healthy human stem cells transduced with C-terminus-truncated CXCR4. Blood 2007;109:78–84.

20 Lagane B, Chow KY, Balabanian K et al. CXCR4 dimerization and beta-arrestin-mediated signaling account for the enhanced chemotaxis to CXCL12 in WHIM syndrome. Blood 2008;112:34–44.

21 Chung E, Hsu CL, Kondo M. Constitutive MAP kinase activation in hematopoietic stem cells induces a myeloproliferative disorder. PLoS One 2011;6:e28350.

22 Kuhn R, Schwenk F, Aguet M et al. Inducible gene targeting in mice. Science 1995;269:1427–1429.

23 Hamanaka S, Ooehara J, Morita Y et al. Generation of transgenic mouse line expressing Kusabira Orange throughout body, including erythrocytes, by random segregation of provirus method. Biochem Biophys Res Commun 2013;435:586–591.

24 Yamamoto R, Morita Y, Ooehara J et al. Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells. Cell 2013; 154:1112–1126.

25 Sanuki S, Hamanaka S, Kaneko S et al. A new red fluorescent protein that allows efficient marking of murine hematopoietic stem cells. J Gene Med 2008;10:965–971.

26 Ema H, Morita Y, Yamazaki S et al. Adult mouse hematopoietic stem cells: Purification and single-cell assays. Nat Protoc 2006;1: 2979–2987.

27 Osawa M, Hanada K, Hamada H et al. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science 1996;273:242–245.

28 Suzuki A, Obi K, Urabe T et al. Feasibility of ex vivo gene therapy for neurological disorders using the new retroviral vector GCDNsap packaged in the vesicular stomatitis virus G protein. J Neurochem 2002;82:953–960.

29 Nabekura T, Otsu M, Nagasawa T et al. Potent vaccine therapy with dendritic cells genetically modified by the gene-silencing-resistant retroviral vector GCDNsap. Mol Ther 2006;13:301–309.

30 Kawai T, Choi U, Whiting-Theobald NL et al. Enhanced function with decreased internalization of carboxy-terminus truncated CXCR4 responsible for WHIM syndrome. Exp Hematol 2005;33:460–468.