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HEMATOPOIESIS AND STEM CELLS

CXCR2 and CXCL4 regulate survival and self-renewal of hematopoietic stem/progenitor cells

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Key Points

- Chemokine ligands CXCL1-4, 6, 10, 11, and 13 are upregulated in human quiescent HSCs with CXCR2 and CXCL4 regulating their survival.
- Genetic ablation of Cxcr2 or Cxcl4 in murine models induces initial expansion but eventual exhaustion of HSC in transplantation assays.

The regulation of hematopoietic stem cell (HSC) survival and self-renewal within the bone marrow (BM) niche is not well understood. We therefore investigated global transcriptomic profiling of normal human HSC/hematopoietic progenitor cells [HPCs], revealing that several chemokine ligands (*CXCL1-4*, *CXCL6*, *CXCL10*, *CXCL11*, and *CXCL13*) were upregulated in human quiescent CD34$^+$Hoescht$^-$Pyronin Y$^-$ and primitive CD34$^+$38$^-$, as compared with proliferating CD34$^+$Hoechst$^+$Pyronin Y$^+$ and CD34$^+$38$^+$ stem/progenitor cells. This suggested that chemokines might play an important role in the homeostasis of HSCs. In human CD34$^+$ hematopoietic cells, knockdown of *CXCL4* or pharmacologic inhibition of the chemokine receptor CXCR2, significantly decreased cell viability and colony forming cell (CFC) potential. Studies on *Cxcr2*$^{-/-}$ mice demonstrated enhanced BM and spleen cellularity, with significantly increased numbers of HSCs, hematopoietic progenitor cell-1 (HPC-1), HPC-2, and Lin$^-$Sca-1$^+$c-Kit$^+$ subpopulations. *Cxcr2*$^{-/-}$ stem/progenitor cells showed reduced self-renewal capacity as measured in serial transplantation assays. Parallel studies on *Cxcl4* demonstrated reduced numbers of CFC in primary and secondary assays following knockdown in murine c-Kit$^+$ cells, and *Cxcl4*$^{-/-}$ mice showed a decrease in HSC and reduced self-renewal capacity after secondary transplantation. These data demonstrate that the CXCR2 network and CXCL4 play a role in the maintenance of normal HSC/HPC cell fates, including survival and self-renewal. (*Blood*. 2016;128(3):371-383)

Introduction

Hematopoietic stem cells (HSCs) are responsible for the maintenance of the hematopoietic system throughout life and their fate is tightly balanced between self-renewal and differentiation in order to sustain the production of multilineage differentiated cells.[1,2] In mice and humans, most adult HSCs are quiescent, yet remain poised for activation in response to bone marrow (BM) injury or growth factor/interferon stimulation.[3,4] This balanced state between quiescence, proliferation, and differentiation is tightly controlled by numerous transcriptional networks, modulated by both cell autonomous factors and the surrounding BM niche.[5]

Chemokines are a family of chemotactic cytokines that bind to specific 7-transmembrane G-protein–coupled receptors.[6] Chemokines are classified into 4 main families based on the position of conserved cysteine residues within their *N*-terminal region; CXC, CC, CX3C, and XC chemokines. Chemokines are functionally divided into 2 groups, homeostatic and inflammatory chemokines. The first are expressed constitutively and are mainly involved in controlling the migration of cells during development and tissue maintenance. Inflammatory chemokines are produced in response to infection or injury and attract inflammatory cells to sites of injury.[7] Chemokines have a variety of roles in the development, disease, and hematopoiesis, in addition to their role in chemotactic activity on leukocytes.[7,8] HSC proliferation and survival have been shown to be mediated by members of the chemokine family.[9,10] Members of the CXC family of chemokines, such as CXCL12 and its receptor CXCR4, play important roles in hematopoietic cell survival, BM localization/retention, and mobilization at early stages of life and during adulthood.[11,12]

The role of chemokine ligands and their receptors in HSC fates has not been extensively characterized so far. We have shown that CCL3 is a negative regulator of HSC proliferation with potential for therapeutic application.[13] A previous study has shown that CXCL1 promotes growth and self-renewal in embryonic stem cells,[14] whereas CXCL4

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has been shown to inhibit cell cycle entry in endothelial cells, together with inhibition of DNA synthesis.[15]

Importantly, recent data have shown that CXCL4 expressed by megakaryocytes (MKs) also regulates HSC quiescence.[16] In addition, CXCL12 and CXCR4 play an important role in maintaining HSC quiescence and repopulating activity.[17,18] HSC survival in vivo is dependent on the expression of *CXCL12* from endothelial cells and mesenchymal progenitors of the surrounding BM stroma.[19] Finally, it has been shown that chemokines support murine leukemia stem cells in myeloproliferative disorders.[20-22]

To identify important regulators of HSC/HPC cell fate decisions, we performed transcriptional profiling of human quiescent and actively dividing stem/progenitor cells, and demonstrated that several chemokine ligands (*CXCL1-4*, *CXCL6*, *CXCL8*, *CXCL10*, *CXCL11*, and *CXCL13*) were upregulated in the quiescent fraction.[23] These data suggested that chemokine signaling pathways may be involved in the regulation of HSC fates, such as survival or self-renewal. Here, we have further investigated and characterized the requirement for members of the chemokine family in mouse and human HSC/HPC cell survival, focusing on CXCR2 and CXCL4.

Materials and methods

Reagents

SB225002 was purchased from Merk (Nottingham, United Kingdom) and used at the concentrations indicated. All reagent grade chemicals were purchased from Sigma-Aldrich (Poole, United Kingdom), unless otherwise stated.

Human samples

This research was approved by the West of Scotland Research Ethics Committee 4 and all human participants gave written informed consent. Granulocyte colony-stimulating factor-mobilized peripheral blood (PB) stem cell samples were obtained as excess material from normal donors for allogeneic transplantation. CD34-enrichment was performed using CliniMACS (Miltenyi Biotec Inc., Auburn, CA) as previously reported.[24] $CD34^+$, $Hoescht^{+/-}$, Pyronin $Y^{+/-}$, and $CD34^+38^{+/-}$ populations were isolated as previously described.[25] Samples were isolated using CD34 (340667; Becton Dickinson, Oxford, United Kingdom) and CD38 (551400; Becton Dickinson) antibodies, Pyronin Y (CAS 92-32-0; San Cruz Biotech, Heidelberg, Germany), and Hoechst (ThermoFisher 33342; Life Technologies, Grand Island, NY).

Mouse strains

All experiments were carried out according to the United Kingdom Home Office regulations using 8- to 12-week-old animals on a C57BL/6 background (CD45.2) with age and sex matched wild-type (WT) controls ($Cxcr2^{-/-}$ and $Cxcl4^{-/-}$). C57BL/6 animals expressing CD45.1 were used as recipient mice for BM reconstitution experiments. The $Cxcl4^{-/-}$ mice were donated by Mortimer Poncz (The Children's Hospital of Philadelphia).

Cell isolation

Human $CD34^+$ cells were cultured in serum free medium supplemented with a high growth factor cocktail as described previously.[23] Murine BM cells were isolated by crushing whole bones. Cells were filtered after crushing. Mouse BM cells were cultured in Iscove modified Dulbecco medium supplemented with 10% fecal calf serum, penicillin, streptomycin, L-glutamine, and interleukin-3 (20 ng/mL), interleukin-6 (20 ng/mL), and stem cell factor (40 ng/mL) (BioLegend, London, United Kingdom). Prior to lentiviral transduction, mouse BM samples were enriched for c-Kit^+ cells using MicroBeads (Miltenyi Biotec Inc.).

Fluorescence-activated cell sorting (FACS)

Flow cytometry was performed using the FACSCantoII and FACSAria cell sorter (Becton Dickinson, Oxford, United Kingdom) and analyzed using FloJo software (Tree Star, Ashland, OR). Murine BM cells were incubated in Fc block prior to antibody staining. Human BM was stained with CD34 (340667; Becton Dickinson) and CD38 (551400; Becton Dickinson). Mouse tissue was stained with a lineage cocktail against CD4 (553649; Becton Dickinson), CD5 (553019; Becton Dickinson), CD8a (553029; Becton Dickinson), Gr-1 (553125; Becton Dickinson), B220 (553086; Becton Dickinson), Ter-119 (553672; Becton Dickinson), and CD11b (553309; Becton Dickinson). The stem/progenitor population was assessed using antibodies against c-Kit (47-1171-82; eBioscience, Hatfield, United Kingdom), Sca-1 (122514; BioLegend), CD150 (115910; BioLegend), and CD48 (103406; BioLegend). For apoptosis assays, cells were stained with Annexin V (556570; Becton Dickinson) and 4,6 diamidino-2-phenylindole (DAPI). Transplantations were monitored using antibodies against CD45.1 and CD45.2 (558701 and 553772, respectively; Becton Dickinson).

RNA isolation and real-time polymerase chain reaction (RT-PCR)

RNA was extracted using the RNeasy RNA Extraction Kit (Qiagen Sciences, Germantown, MD) and reverse transcribed into complementary DNA (Life Technologies). If <5000, cells were sorted using the CellsDirect One-Step qRT-PCR Kit (Life Technologies, Paisley, United Kingdom). RT-PCR was carried out using TaqMan probes and data were acquired using an Applied Biosystems 7900HT Fast RT-PCR machine (Life Technologies) or Fluidigm platform (Fluidigm Corporation), and data analysis was performed using the Relative Quantitation Manager Software (Life Technologies). Human TaqMan probe ID numbers are as follows: human *CXCL1*: Hs00236937_m1; human *CXCL2*: Hs00601975_m1; human *CXCL4*: Hs00427220_g1; human *CXCL6*: Hs00605742_g1; human *CDC6*: Hs00153374_m1; human *CD38*: Hs01120071_m1; and human *CXCL8*: Hs01115388_m1. Mouse *Cxcr2*: Mm00438258_m1; mouse *Cxcl4*: Mm00451315_g1; mouse *Cxcl1*: Mm04207460_m1; mouse *Cxcl2*: Mm00436450_m1; mouse *Cxcl3*: Mm01701838_m1; and mouse *Cxcl12*: Mm00445553_m1.

Immunofluorescence staining

Cells were fixed and permeabilized with 4% formaldehyde and 0.25% Triton X-100, blocked in 10% goat or donkey serum (Sigma-Aldrich) and incubated overnight in primary antibodies against CXCL1 (1:100, sc-1374; Santa Cruz, Heidelberg, Germany), CXCL2 (1:100, sc-1375; Santa Cruz), CXCL4 (1:100, sc-73638; Santa Cruz), CXCL6 (1:100, sc-5813; Santa Cruz), CXCL8 (1:100, MAB208; R&D Systems, Abingdon, United Kingdom), CXCR2 (1:100, sc-682; Santa Cruz), or immunoglobulin G (AB-108-C; R&D Systems). Cells were incubated with appropriate secondary donkey anti-goat (1:500, ab150129; Abcam, Cambridge, United Kingdom), goat anti-mouse (1:500, ab150113; Abcam), or goat anti-rabbit (1:500, ab150077; Abcam), Alexa Fluor 488, and mounted with DAPI (Vector Laboratories Ltd, Burlingame, CA). Images were acquired using a Zeiss fluorescence microscope and 3-dimensional images were generated using ImageJ.

Colony forming cell (CFC) assay

CFC assays were carried out using MethoCult (H4434 or M3434; Stem Cell Technologies, Grenoble, France). Resulting colonies were counted after ~10 to 14 days in culture. Colonies were harvested, counted, and reseeded into MethoCult for serial replating assays.

In vivo transplantation

Donor mice were $CD45.2^+$. HSC ($Lin^-Sca\text{-}1^+c\text{-}Kit^+$ [LSK], $CD150^+CD48^-$) were sorted from donor $Cxcr2^{-/-}$, $Cxcl4^{-/-}$, or WT animals, and transplanted by IV injections into $CD45.1^+$ recipient mice (irradiated at 7 Gy) using 100 HSC together with 200 000 $CD45.1^+$ support BM cells per mouse. Mice were given Baytril antibiotic in their drinking water for 2 weeks following irradiation. Animals were bled every 4 weeks posttransplant to assess chimerism and sacrificed at 16 weeks posttransplant. BM, spleen, and thymus were harvested

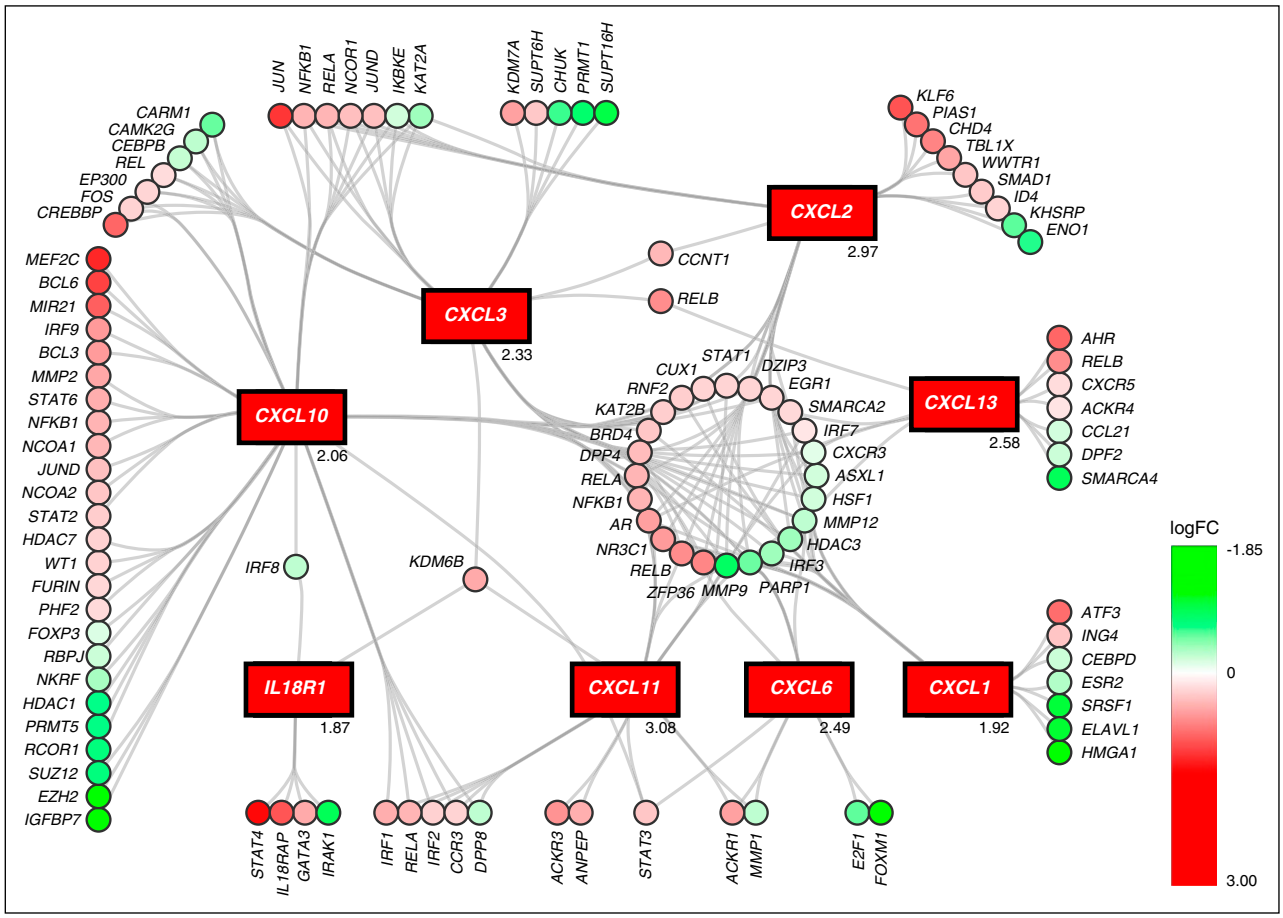

Figure 1. Chemokine ligands are upregulated in quiescent vs dividing CD34$^+$ cells. The transcriptional differential regulation connected to chemokine expression (shown in rectangles) in human quiescent (CD34$^+$, Hoechst$^-$, and Pyronin Y$^-$) vs dividing (CD34$^+$, Hoechst$^+$, and Pyronin Y$^+$) cells. Upregulation is shown in red and downregulation in green; color intensity indicates the extent of differential regulation as indicated in the color key. logFC values for the chemokines are given on the bottom-right of the relevant box. logFC, log fold change.

and examined for donor cell contribution. For secondary transplantations, 2000 CD45.2$^+$ LSK cells were sorted from primary recipients, and transplanted with 200 000 CD45.1$^+$ support BM cells into CD45.1$^+$ irradiated recipient mice and followed as above. For homing experiments, sorted LSK donor cells (1×10^5) were transplanted into lethally irradiated recipient mice; 24 hours after transplant, the recipient mice were analyzed.

Lentiviral transduction and electroporation

Mouse Cxcl4 short hairpin RNA (shRNA) (Cat No. RMM4534-NM_019932) were purchased from Thermo Fisher (Life Technologies). shRNA of interest were subcloned from the original pLKO.1 vectors into a pLKO.1 plasmid containing a green fluorescent protein (GFP) tag. Calcium chloride was used to transfect human embryonic kidney-293 cells with specific hairpin or scrambled control using HIV-1 and glycoprotein of vesicular stomatitis virus as accessory plasmids. Primary cells were cultured in viral medium supplemented with TransDux (Cambridge Bioscience, Cambridge, United Kingdom) or Polybrene at 400*g* for 90 minutes. Viral medium was collected at 24, 48, and 72 hours posttransfection, and the spin inoculation was repeated for 3 rounds of viral infection. Cells were resuspended in appropriate medium for 48 hours before selection of positively transduced cells using positive expression of GFP and FACS.

CXCL4-small interfering RNA (siRNA) (221753; Applied Biosystems) and scrambled siRNA1 (AM4611; Applied Biosystems) (100 nM) were electroporated into cells using the Amaxa Nucleofector Kit V (Lonza, Cambridge, United Kingdom) together with a GFP-containing plasmid following the manufacturer's instructions. Transduction efficiency ranged between 30% and 50%. After 24 hours, the GFP$^+$ cells were sorted and analyzed.

Differential expression analysis of transcriptional data and network construction

Raw data were obtained via ArrayExpress (E-MTAB-2508); robust multi-array analysis was normalized and analyzed using Rank products.[26,27] MetaCore's "Expand by one interaction" algorithm was used to expand the gene network around the 8 chemokines differentially expressed in quiescent vs dividing CD34$^+$ cells[23]; only those genes exhibiting differential expression (false discovery rate = 0.05) between quiescent and dividing normal CD34$^+$ cells were retained.

Statistical analysis

Statistical analyses were performed using the Student *t* test. $^*P < .05$ was defined as statistically significant. $^{**}P < .01$ and $^{***}P < .001$ were considered highly statistically significant.

Results

Chemokine ligands are upregulated in human CD34$^+$38$^-$ compared with CD34$^+$38$^+$ cells

We previously analyzed the transcriptional differences between human quiescent (CD34$^+$, Hoechst$^-$, and Pyronin Y$^-$) cells and their more proliferative (CD34$^+$, Hoechst$^+$, and Pyronin Y$^+$) counterparts. We observed that several chemokine ligands, including *CXCL1-3*, *CXCL6*,

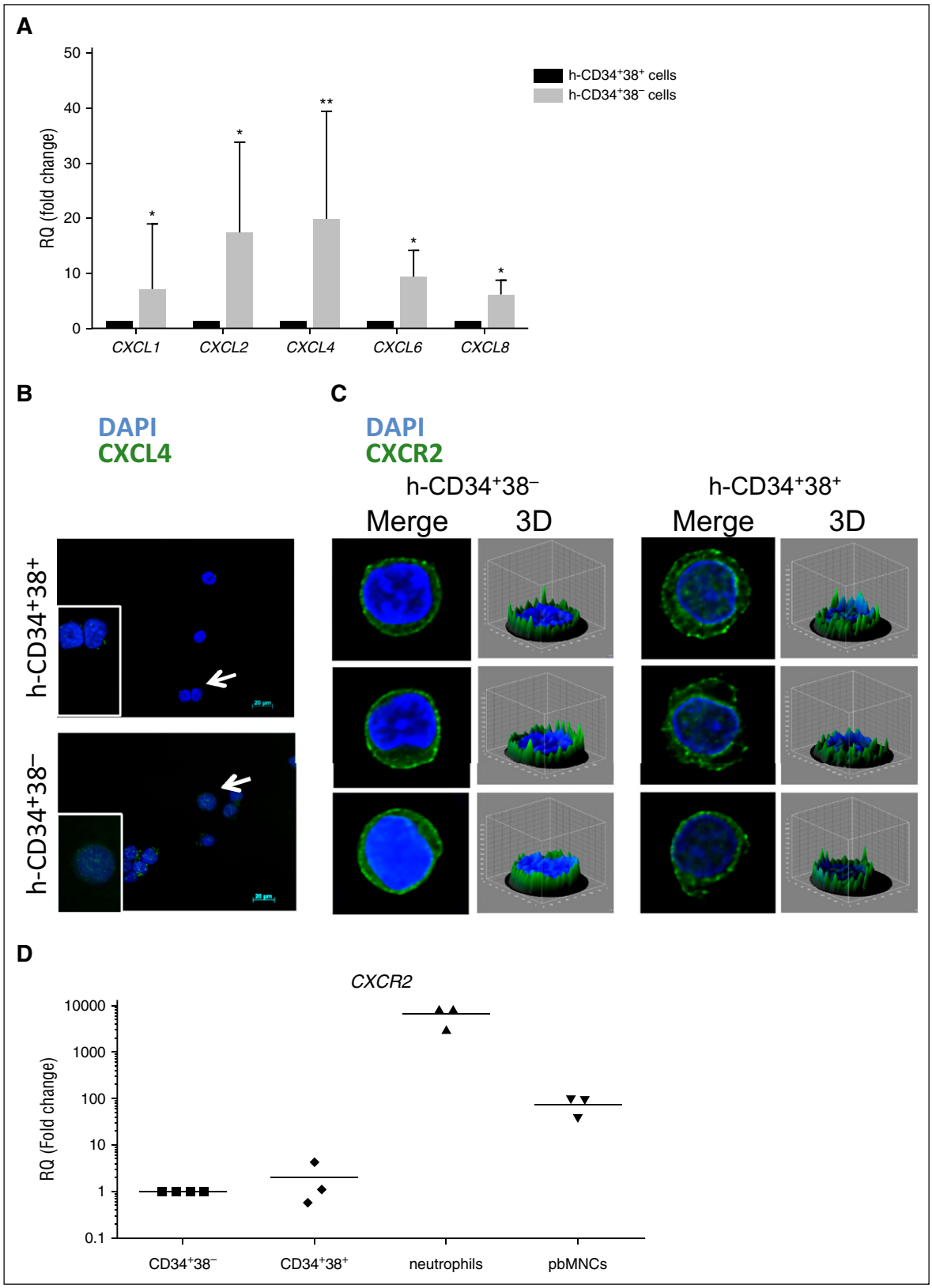

Figure 2. CXCL4 and CXCR2 are expressed on CD34$^+$38$^-$ and CD34$^+$38$^+$ cells. (A) RT-PCR in human CD34$^+$38$^-$ and CD34$^+$38$^+$ cells shows gene expression differences for the chemokine ligands *CXCL1*, *CXCL2*, *CXCL4*, *CXCL6*, and *CXCL8*. Fold change was calculated relative to the reference gene (*GAPDH*) according to the ΔΔCT method. n = 5. **P* < .05; ***P* < .01. All error bars indicate standard error of the mean (SEM). (B) Human CD34$^+$38$^-$ (top) and CD34$^+$38$^+$ (bottom) cells show expression for CXCL4 (white arrows indicate cells in enlarged picture), and (C) CXCR2. Nuclei were stained using DAPI and images were acquired using a Zeiss microscope; 3-D figures generated with ImageJ software are shown for CXCR2 staining (n = 3). (D) Real-time quantitative PCR in human CD34$^+$38$^-$, CD34$^+$38$^+$, neutrophils, and PB mononuclear cells shows the gene expression profile for *CXCR2* (n = 3). The fold change was calculated relative to the reference gene (*GAPDH*) according to the ΔΔCT method. 3-D, 3-dimensional; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; h, human; RQ, relative quantitation.

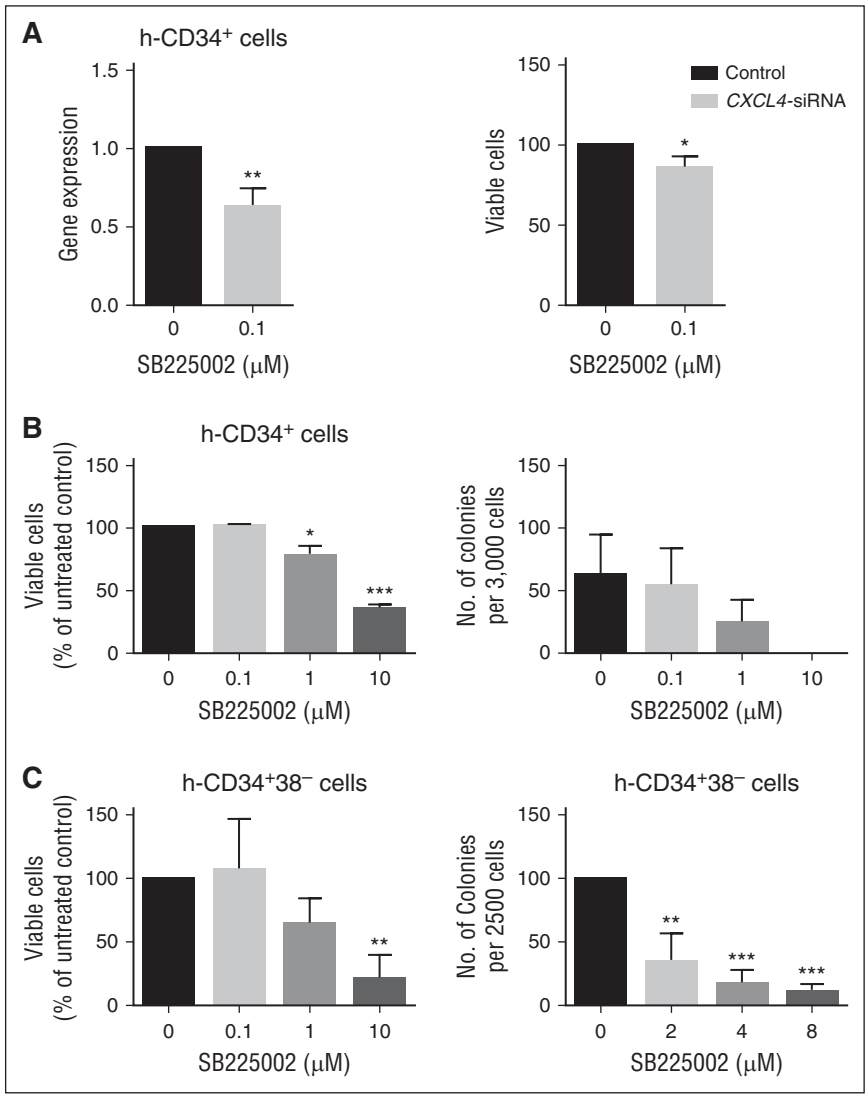

Figure 3. Inhibition of *CXCL4* ligands and CXCR2 signaling reduces cell viability and colony formation in vitro. (A) $CD34^+$ enriched cells were transduced with *CXCL4* siRNA, and relative scrambled control and level of knock-down (left) and viability (right) measured (n = 3). (B) $CD34^+$ cells were treated with increasing concentrations of SB225002 and viable cells analyzed using Annexin V/DAPI staining (left) (n = 3). CFC count was performed in the same cells treated with SB225002 at the concentration indicated (right) (n = 3). (C) $CD34^+38^-$ enriched cells were treated with increasing concentrations of SB225002 and viable cells analyzed using Annexin V/DAPI staining (left) (n = 3). CFC count was performed in cells treated with SB225002 at the concentration indicated (right) (n = 3). Statistical analysis was performed using a paired two-tailed Student *t* test (*$P < .05$; **$P < .01$; ***$P < .001$). All error bars indicate SEM of the mean. h, human.

CXCL10, *CXCL11*, and *CXCL13* were upregulated in the quiescent population, suggesting a possible role for these factors in the regulation of HSC maintenance.[23] No probes for *CXCL4* were included in the microarray. Here we have further analyzed these data with a focus on the chemokine family. Figure 1 shows the upregulated chemokines and genes predicted to be connected with the chemokine network in human HSCs. In quiescent cells, the chemokine network highlighted regulation of factors relevant for proliferative status, such as regulators of cell cycle E2F1 (downregulated) and EGR1 (upregulated).[28] To validate chemokine regulation and examine a possible mechanism by which these ligands are linked to HSC cell cycle status, BM stem/progenitor cells from normal, healthy volunteers were sorted into primitive ($CD34^+38^-$) and more proliferative progenitor cell ($CD34^+38^+$) populations. Cell cycle and differentiation status of these 2 populations was confirmed by the differential expression of *CDC6* and *CD38*, respectively (see supplemental Figure 1, available on the *Blood* Web site). As predicted, *CDC6* and *CD38* were downregulated in $CD34^+38^-$ compared with $CD34^+38^+$ cells, indicating that $CD34^+38^-$ cells were mainly quiescent and undifferentiated. Chemokine genes were selected for RT-PCR validation based on the level of upregulation in the microarray with the addition of *CXCL4*, which has recently been shown to be relevant in the regulation of HSC quiescence.[16] The expression of *CXCL1*, *CXCL2*, *CXCL4*, *CXCL6*, and *CXCL8* was significantly increased in $CD34^+38^-$ compared with $CD34^+38^+$ cells (Figure 2A; *$P < .05$; **$P < .01$), suggesting a possible role for these chemokines in maintenance of the quiescent HSC pool.

Immunofluorescence staining showed that human CXCL4 protein was detectable in both $CD34^+38^-$ and $CD34^+38^+$ cells, but was higher in expression in $CD34^+38^-$ cells (Figure 2B). Similarly, immunofluorescence staining was carried out in human $CD34^+38^+$ and $CD34^+38^-$ cells to investigate the expression of CXCL1, CXCL2, CXCL6, and CXCL8 (supplemental Figure 1B). Although CXCL6 and CXCL8 were barely detectable at the protein level in either population, CXCL1 and CXCL2 showed clear expression in $CD34^+38^+$ cells, but in contrast to what was observed at the gene level, no upregulation was detected in the $CD34^+38^-$ cells. Because CXCR2 is the best characterized of the receptors for chemokine ligands, we examined its expression at the messenger RNA (mRNA) (Figure 2D) and protein level (Figure 2C and supplemental Figure 1B) in the $CD34^+38^-$ and $CD34^+38^+$ fractions. CXCR2 mRNA and protein were expressed at similar levels in both cell populations. The *CXCR2* mRNA level was compared with that of neutrophils and PB mononuclear cells that were used as positive controls. Based on their roles in cell growth or self-renewal in other cellular contexts, *CXCL4* and CXCR2 were selected for functional studies.[15,16]

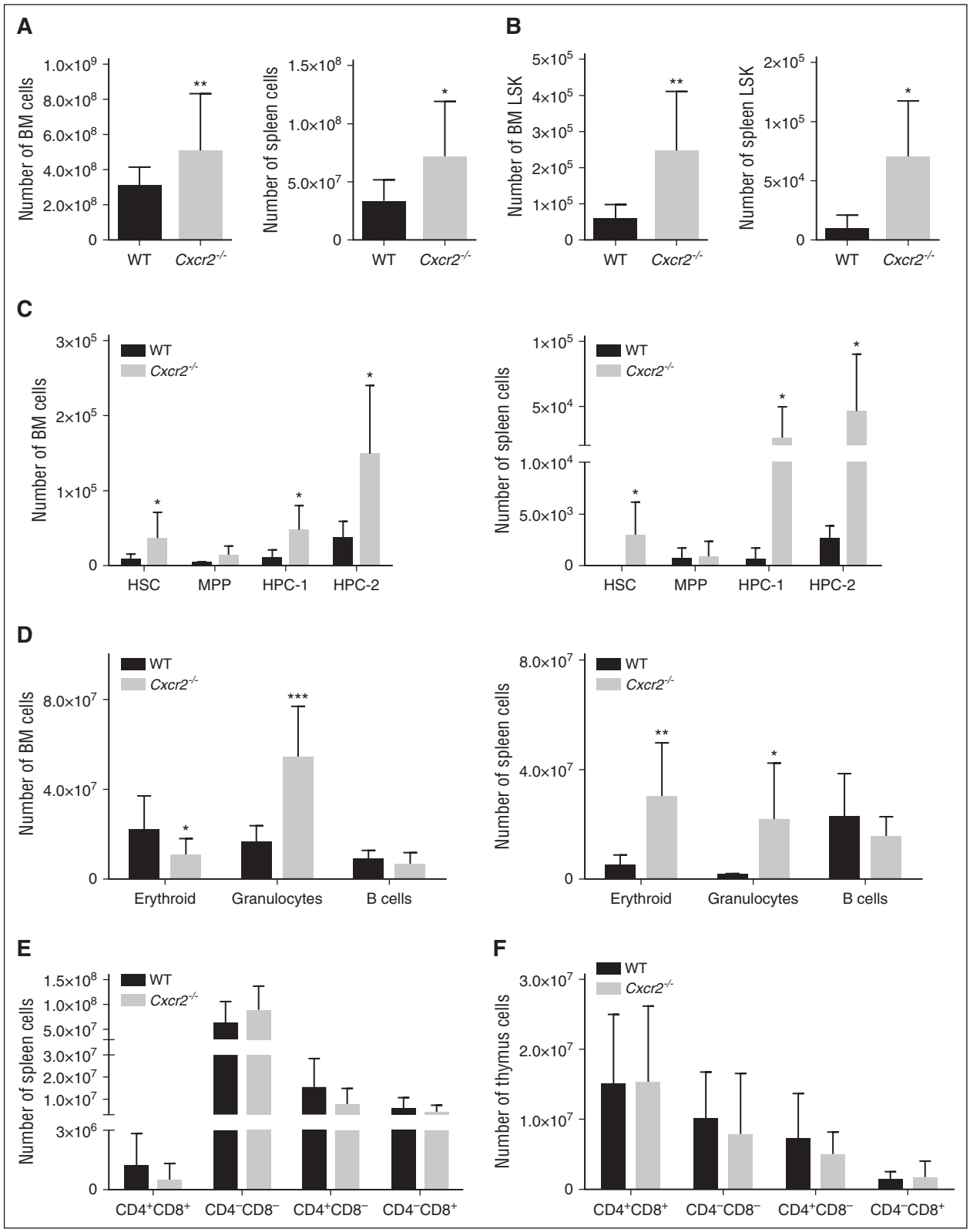

Figure 4. *Cxcr2*$^{-/-}$ mice show an expansion of the stem cell compartment. (A) BM (left) and spleen (right) from WT mice (n = 6) or *Cxcr2*$^{-/-}$ mice (n = 6) were harvested and assessed for total cellularity. (B) Numbers of LSK cells for BM (left) and spleen (right) from WT mice or *Cxcr2*$^{-/-}$ mice. (C) Numbers of HSC, MPP, HPC-1, and HPC-2 in the BM (left) and spleen (right) from WT mice or *Cxcr2*$^{-/-}$ mice are shown. (D) Numbers for BM (left) and spleen (right) erythroid, granulocytic, and B-cell populations from WT and *Cxcr2*$^{-/-}$ mice are shown. (E) Numbers for T-cell populations in the WT and *Cxcr2*$^{-/-}$ spleen identified with CD4 and CD8 markers are shown. (F) Numbers for T-lineage populations in the WT and *Cxcr2*$^{-/-}$ thymi identified with CD4 and CD8 markers are shown. Statistical analysis was performed using an unpaired two-tailed Student *t* test. All error bars indicate SEM of the mean (*$P < .05$; **$P < .01$; ***$P < .001$) (n = 6).

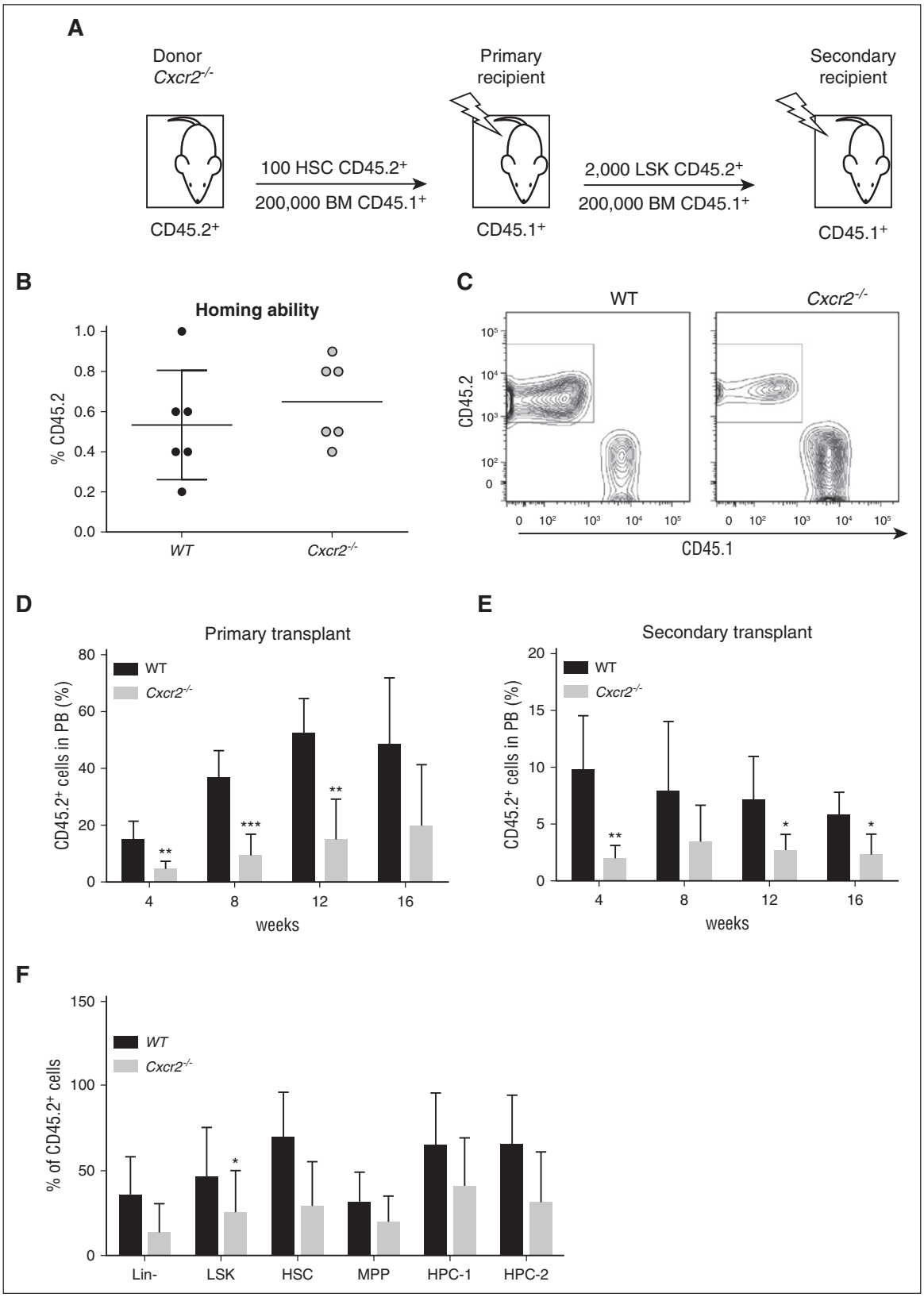

Figure 5. *Cxcr2*$^{-/-}$ HSCs show a reduction in engraftment in primary and secondary BM transplantation assays. (A) Experimental layout for CD45.2$^+$ HSCs from WT or *Cxcr2*$^{-/-}$ mice (n = 3 per strain) transplanted into irradiated CD45.1$^+$ recipients (n = 6). (B) Graph showing engraftment ability of CD45.2$^+$ WT or *Cxcr2*$^{-/-}$ HSC after 24 hours from transplant in CD45.1$^+$ recipients. (C) Graph showing chimerism between CD45.2$^+$ and CD45.1$^+$ cells. (D) Engraftment was analyzed in the blood every 4 weeks posttransplant up to 16 weeks. Data are presented as the mean percentage of CD45.2$^+$ cells within the PB. (E) After the primary recipients were sacrificed, CD45.2$^+$ LSK cells were transplanted into irradiated recipients. Engraftment was analyzed in the blood every 4 weeks posttransplant up to 16 weeks. (F) Percentage of CD45.2$^+$ cells was analyzed in different BM populations after 16 weeks from primary transplant, and compared between WT and *Cxcr2*$^{-/-}$ mice. Statistical analysis was performed using an unpaired two-tailed Student *t* test. All error bars indicate SEM of the mean (*$P < .05$; **$P < .01$; ***$P < .001$) (n = 6).

CXCL4 and CXCR2 support viability and colony forming potential of human CD34$^+$ cells

We investigated the role of the most differentially expressed chemokine *CXCL4*, in CD34$^+$ cells. In keeping with the <50% reduction in gene expression achieved, siRNA-mediated knock-down of *CXCL4* resulted in a modest but significant decrease in cell viability compared with nontargeting siRNA (Figure 3A). To investigate the role of CXCL4's known chemokine receptor CXCR2, CD34$^+$ cells were treated with the CXCR2 inhibitor SB225002. SB225002 is a potent and selective antagonist of CXCR2, which results in inhibition of ligand-mediated signal transduction.[29] CD34$^+$ cells were treated for 72 hours. A reduction in cell viability and colony formation was observed in a drug concentration-dependent manner compared with the vehicle-treated control (Figure 3B) (drug concentration based on published literature[30]). Similarly, CD34$^+$38$^-$ cells treated for 72 hours with SB225002 showed a marked decrease in cell viability and colony formation ability in comparison with control (Figure 3C). In addition, treatment of CD34$^+$38$^-$ cells with SB225002 at 1 μM led to an obvious increase in the percentage of cells in gap 0 (G0) of the cell cycle (supplemental Figure 1C). Taken together, these data suggest that inhibition of chemokine signaling, in particular that of *CXCL4* and *CXCR2*, impairs human stem/progenitor cell function, and imply that these factors may be required for survival and maintenance of these cells.

Chemokine expression in murine stem and progenitor cells

To investigate a role for the chemokine family in the maintenance of HSCs in their physiological BM microenvironment, parallel murine models were employed. We first interrogated a publicly available gene expression data set and determined that *Cxcl4* was expressed in murine LSK cells.[31] Following cell sorting, real-time quantitative PCR was used to assess the expression of *Cxcl1*, *Cxcl2*, *Cxcl3*, *Cxcl4*, *Cxcl12*, and *Cxcr2* across HSC (LSKCD150$^+$CD48$^-$), multipotent progenitors (MPPs; LSKCD150$^-$CD48$^-$), and primitive HPCs (HPC-1; LSKCD150$^-$CD48$^+$ and HPC-2; and LSKCD150$^+$CD48$^+$) derived from adult murine BM (see sorting strategy in supplemental Figure 2A; data in supplemental Figure 2B; and Calaminus et al[32]). As shown, *Cxcl4* and *Cxcr2* were expressed in the HSC fraction.

Cxcr2$^{-/-}$ HSCs have impaired self-renewal capacity

We next investigated the requirement for *Cxcr2* in mouse HSC function. *Cxcr2*$^{-/-}$ mice have previously been characterized and are known to show a considerable expansion of myeloid cells in the BM, spleen, and PB, an effect that was shown to be caused by the deletion of *Cxcr2* from the BM microenvironment.[33,34] However, to our knowledge, a comprehensive analysis of the hematopoietic stem and progenitor compartments, and their functionality, has not previously been investigated in these mice. Total BM and spleen cellularity were increased in the *Cxcr2*$^{-/-}$ mice (*$P < .05$; **$P < .01$) (Figure 4A). Immunophenotypic analyses revealed that the absolute numbers of LSK cells were increased in the BM and spleen of *Cxcr2*$^{-/-}$ mice (*$P < .05$; **$P < .01$) (Figure 4B). More detailed analyses of the LSK compartment using CD150 and CD48 markers indicated a significant increase in the HSC, HPC-1, and HPC-2 populations in the *Cxcr2*$^{-/-}$ mice, both in the BM and in the spleen (*$P < .05$) (Figure 4C). A decrease in the erythroid (*$P < .05$) and an increase in the myeloid compartments (***$P < .001$) of *Cxcr2*$^{-/-}$ mice were also observed in the BM, consistent with the previous report (Figure 4D, left).[34] In the spleen of *Cxcr2*$^{-/-}$ mice, an increase in both erythroid and the granulocytic compartments was seen (*$P < .05$; **$P < .01$) (Figure 4D, right). No differences were detected in the T-cell compartments (CD4 and CD8) between the *Cxcr2*$^{-/-}$ and the WT mice, either in the spleen or in the thymus (Figure 4E-F). When cell cycle was analyzed in long-term HSCs from WT and *Cxcr2*$^{-/-}$ mice, a decrease in the percentage of *Cxcr2*$^{-/-}$ cells in G0 phase, together with an increase in those in G1 phase was detected (supplemental Figure 3A). No changes in apoptosis were detected (supplemental Figure 3C).

In previous examples of genes that are critical for HSC maintenance, an expansion in stem and progenitor cell numbers has often been associated with an alteration in the balance between self-renewal and differentiation, leading to stem cell exhaustion.[35] To determine whether the difference observed in overall HSC frequency in *Cxcr2*$^{-/-}$ mice translated into a change in self-renewal capacity, *Cxcr2*$^{-/-}$ HSCs were investigated in serial transplantation assays (Figure 5A). No difference in homing ability was detected between the WT and *Cxcr2*$^{-/-}$ HSCs at 24 hours following transplantation (Figure 5B). HSCs from WT or *Cxcr2*$^{-/-}$ mice (CD45.2 background) were transplanted into irradiated recipients (CD45.1 background), together with CD45.1$^+$ support BM cells. Chimerism between donor CD45.2 and recipient CD45.1 was analyzed (Figure 5C). A decrease in the percentage of CD45.2$^+$ chimerism was observed in the PB up to 16 weeks posttransplant for *Cxcr2*$^{-/-}$ HSCs in comparison with the control (**$P < .01$; ***$P < .001$) (Figure 5D). To further investigate the self-renewal capacity of *Cxcr2*$^{-/-}$ HSCs, secondary transplantation assays were carried out at 16 weeks post-primary transplant. BM from the recipients of the primary transplants was harvested and donor-derived LSK were transplanted into irradiated secondary recipients together with support BM. A decrease in the percentage of donor-derived cells was found in the PB of secondary recipients transplanted with *Cxcr2*$^{-/-}$ LSK in comparison with the controls out to 16 weeks (**$P < .01$; *$P < .05$) (Figure 5E). To elucidate whether the phenotype observed in *Cxcr2*$^{-/-}$ mice was due to an HSC autonomous effect or conferred by the surrounding niche (non-autonomous effect), we analyzed the phenotype of donor-derived BM cells after primary transplantation in WT recipient mice (Figure 5F). In contrast to what was observed in mutant LSK prior to transplant (significant increase as compared with WT; Figure 4B), a significant decrease was observed in the percentage of donor mutant LSK compared with the WT following transplantation, suggesting that signals from the microenvironment may play a role in the phenotype observed. Collectively, the results show that *Cxcr2* plays a key role in HSC maintenance.

Cxcl4 contributes to the regulation of self-renewal of HSCs and progenitor cells

To support the in vitro data on human CXCL4 (Figures 1-3) and to complement the *Cxcr2*$^{-/-}$ mouse model, we analyzed hematopoiesis in *Cxcl4*$^{-/-}$ mice.[36] Previous studies have shown that *Cxcl4*$^{-/-}$ mice exhibited an increased number of HSC and increased HSC proliferation.[16] We observed that *Cxcl4*$^{-/-}$ mice showed normal spleen and BM cellularity (Figure 6A), whereas the LSK numbers in BM were increased (*$P < .05$) (Figure 6B). Furthermore, we found that *Cxcl4*$^{-/-}$ mice had a significant decrease in the HSC and an increase in MPP in the BM, but not in the spleen (*$P < .05$; ***$P < .001$) (Figure 6C). Mature cell subsets, including erythroid, granulocytic, and B and T cells, were not significantly altered in the BM, spleen, or thymus of *Cxcl4*$^{-/-}$ mice (Figure 6D-F). When cell cycle (LSK cells) was analyzed in the WT and *Cxcl4*$^{-/-}$ mice, no significant difference was detected between phases (supplemental Figure 3B). No changes in apoptosis were detected (supplemental Figure 3D).

To investigate changes in self-renewal capacity, a specific *Cxcl4* shRNA construct was used to reduce *Cxcl4* expression in mouse c-Kit$^+$ BM cells using a lentiviral transduction system. A reduction in *Cxcl4*

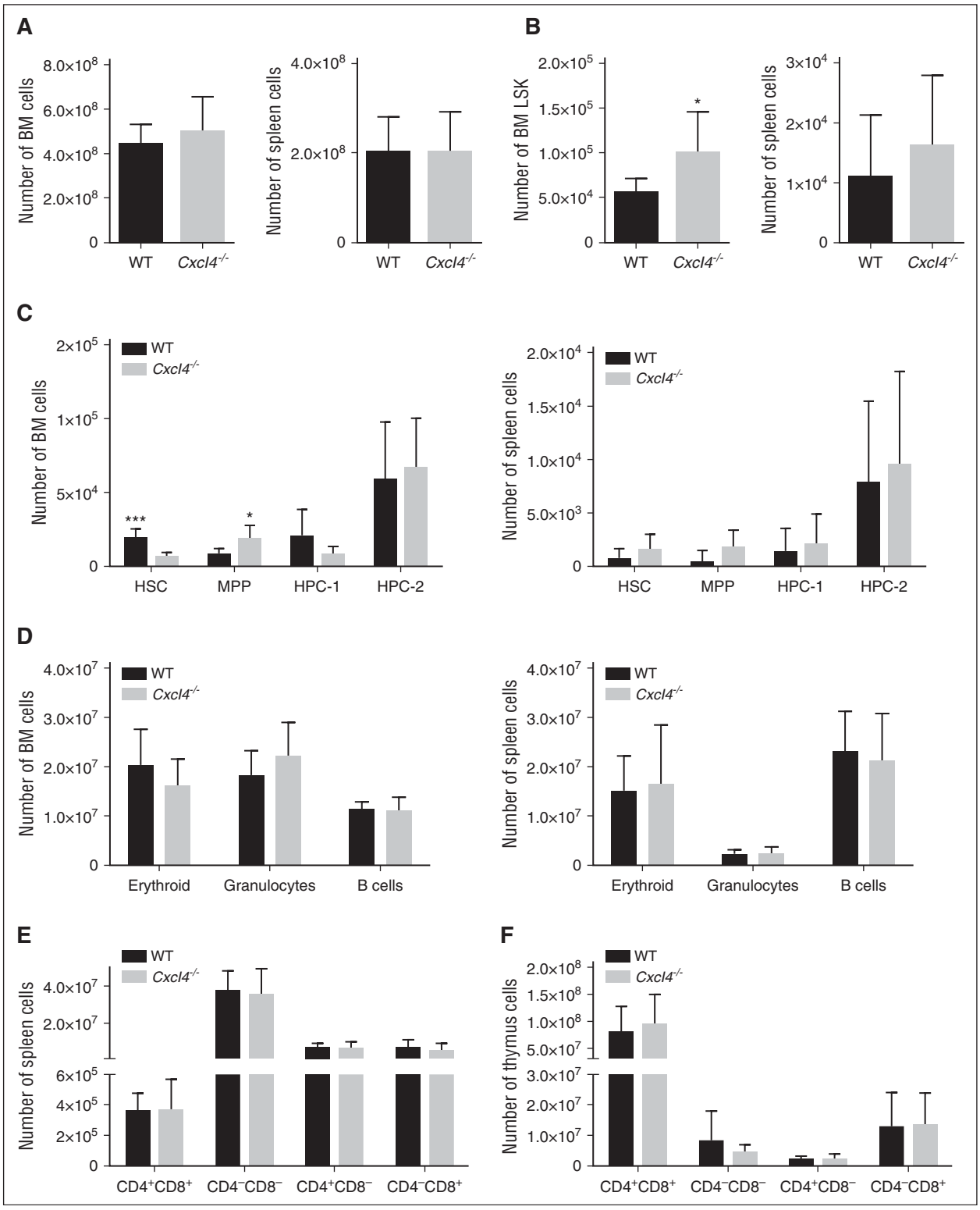

Figure 6. *Cxcl4*$^{-/-}$ mice display a reduction in HSC compartment. (A) BM (left) and spleen (right) from WT mice (n = 6) or *Cxcl4*$^{-/-}$ mice (n = 6) were harvested and total cellularity assessed. (B) Numbers of LSK cells for BM (left) and spleen (right) from WT mice or *Cxcl4*$^{-/-}$ mice. (C) Numbers of HSC, MPP, HPC-1, and HPC-2 in the BM (left) and spleen (right) from WT mice or *Cxcl4*$^{-/-}$ mice are shown. (D) Numbers for BM (left) and spleen (right) erythroid, granulocyte, and B-cell compartments from WT and *Cxcl4*$^{-/-}$ mice are shown. (E) Numbers for T-cell populations in the WT and *Cxcl4*$^{-/-}$ spleen identified with CD4 and CD8 markers are shown. (F) Numbers for T-lineage populations in the WT and *Cxcl4*$^{-/-}$ thymi were identified with CD4 and CD8 markers. Statistical analysis was performed using an unpaired two-tailed Student *t* test. All error bars indicate SEM of the mean (*$P < .05$; ***$P < .001$).

gene expression was found in *Cxcl4* knockdown cells (control, n = 2; *Cxcl4*-sh1, n = 3) (Figure 7A). To determine whether *Cxcl4* played a role in progenitor cell self-renewal, *Cxcl4* knockdown cells were plated into methylcellulose and then replated after 7 days. A significant reduction in CFC was detected in both the primary (***$P < .001$) and secondary cultures (*$P < .05$) (Figure 7B-C). *Cxcl4*$^{-/-}$ HSCs were

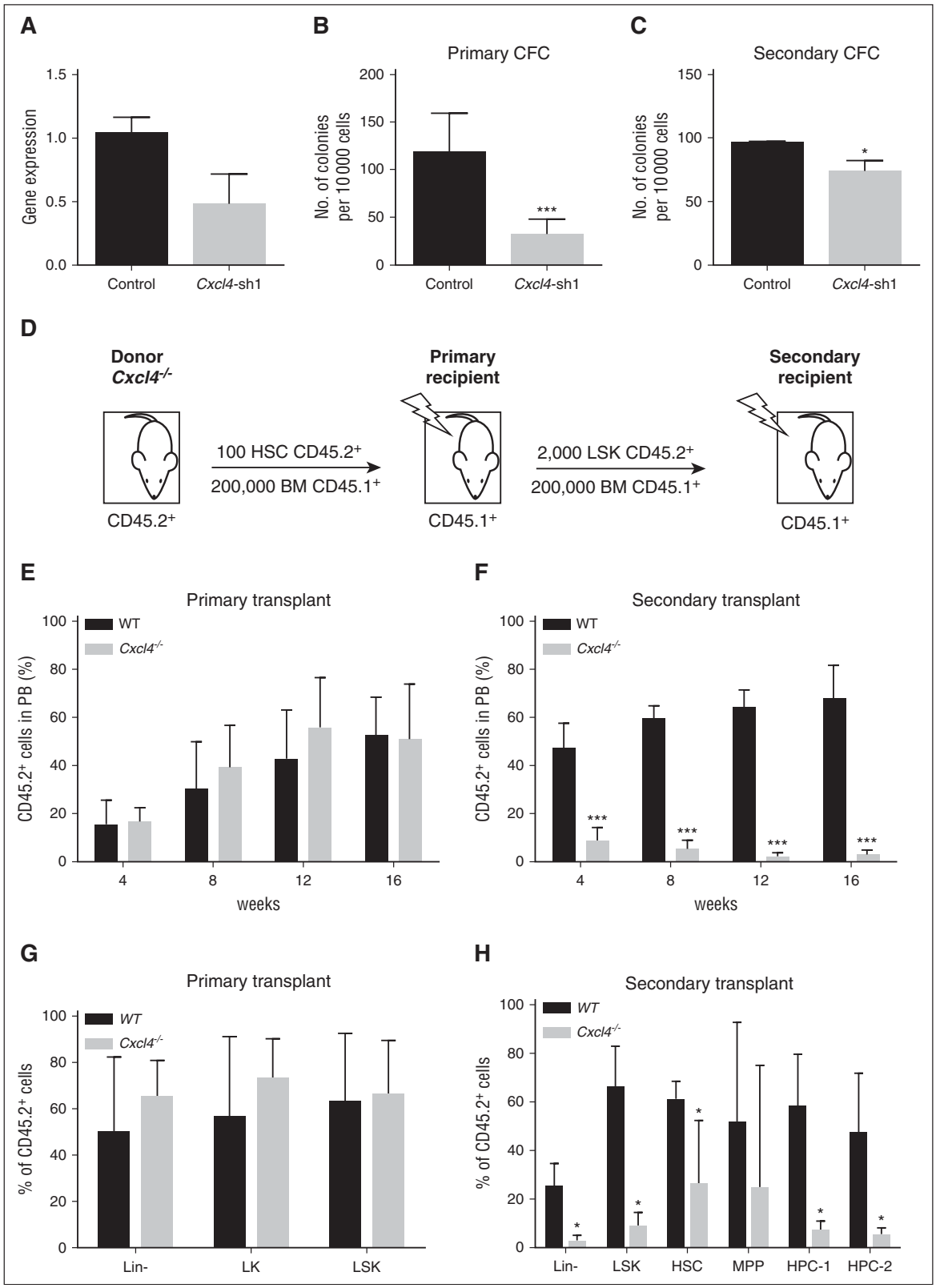

Figure 7. Inhibition of *Cxcl4* reduces colony formation in vitro and *Cxcl4*$^{-/-}$ HSCs show a reduction in engraftment in secondary BM transplantation assays. (A) WT BM cells enriched for c-Kit^{+} were transduced with a *Cxcl4*-shRNA vector or control, and mRNA level analyzed by RT-PCR (n = 2). (B-C) Positively transduced cells were selected using GFP, and plated into primary (B) and secondary (C) CFC assays. Data are presented as the mean colony numbers from *Cxcl4*-shRNA transduced cells or the control. (D) Experimental layout for CD45.2^{+} HSCs from WT or *Cxcl4*$^{-/-}$ mice transplanted into irradiated CD45.1^{+} recipients (n = 5 per strain). (E) Engraftment for primary transplant was analyzed in the blood every 4 weeks posttransplant up to 16 weeks. (F) After the primary recipients were sacrificed, CD45.2^{+} LSK cells were transplanted into irradiated recipients (n = 5). Engraftment was analyzed in the blood every 4 weeks posttransplant up to 16 weeks. (G) A percentage of CD45.2^{+} cells was analyzed in different BM populations after 16 weeks from primary transplant, and compared between WT and *Cxcl4*$^{-/-}$ mice. (H) Percentage of CD45.2^{+} cells was analyzed in different BM populations after 16 weeks from secondary transplant, and compared between WT and *Cxcl4*$^{-/-}$ mice. Data are presented as the mean percentage of CD45.2^{+} cells within the PB. *$P < .05$; ***$P < .001$.

then investigated for their ability to reconstitute myeloablated hosts in primary and secondary transplantation assays, as previously carried out for *Cxcr2*$^{-/-}$ HSCs (Figures 5 and 7D). No changes in the percentage of CD45.2^{+} donor chimerism were observed in the PB up to 16 weeks post-primary transplant for the *Cxcl4*$^{-/-}$ HSCs in comparison with the control (Figure 7E). However, secondary transplantation assays showed a significant decrease in the percentage of donor-derived cells in the PB of recipients transplanted with *Cxcl4*$^{-/-}$ LSK in comparison with the controls out to 16 weeks (***$P < .001$) (Figure 7F), indicating that *Cxcl4* also contributes to HSC maintenance.

Similar to the analysis performed for *Cxcr2*$^{-/-}$, we analyzed the phenotype of *Cxcl4*$^{-/-}$ donor-derived BM cells after primary transplantation into WT recipient mice, but no difference was seen between the WT and the mutant percentage of donor cells (Figure 7G). However, significant decreases were observed in the percentages of donor mutant cells after secondary transplantation in all populations except MPP, when compared with the WT counterparts (Figure 7H). In this case, the effect observed in the *Cxcl4*$^{-/-}$ HSCs before transplantation (significant decrease as compared with WT; Figure 6C) was maintained after transplantation into a WT microenvironment.

Discussion

Our results indicate that chemokines play an important role in the regulation of HSC survival and self-renewal. HSC homeostasis is a critical process required for the correct functioning of HSCs and their progeny. Here we propose that chemokine signaling pathways are involved in the regulation of HSCs, and cooperate to maintain the quiescent and self-renewing state typical of these cells. Our findings suggest that some chemokines, in particular CXCL4 and the receptor CXCR2, play a key role in the maintenance of the HSC pool. We have observed that *Cxcr2* and *Cxcl4* proved to be critical for HSC reconstitution, suggesting a role in self-renewal.

CXCL1-4, together with CXCL6, CXCL8, CXCL10, CXCL11, and CXCL13, were all significantly upregulated in the quiescent/primitive fraction of human stem/progenitor cells and, upon modest knockdown of CXCL4, the survival of primitive cells was impaired. It has been shown previously that human hematopoietic CD34^{+} cells and endothelial cells respond to exogenous CXCL4, with effects on cell viability, adhesion, and stem cell expansion.[15,37-40]

CXCL4 signaling is complex and in certain biological contexts its functions appear to be mediated by the alternatively spliced receptor CXCR3 (CXCR3B).[41] However, to date, expression of CXCR3B has not been described on human or murine HSCs. It has also been suggested that CXCL4 functions may be mediated through another, unnamed receptor/mechanism, with CXCL4 binding to integrin receptors, which have indeed been shown to be important for HSC behavior.[42]

Interactions between CXCL12 and CXCR4 are of key importance for the maintenance of HSCs in humans.[41-43] AMD3100, a selective CXCR4 antagonist (plerixafor), antagonizes the binding of CXCL12 to CXCR4, leading to a rapid and reversible mobilization of HSCs into the peripheral circulation.[44] Currently, plerixafor is used for mobilization of stem cells in patients with non-Hodgkin lymphoma or multiple myeloma.[45-47]

It has been shown that microenvironmental Cxcl4 derived from MKs regulates HSC cell cycle activity. Using transgenic inducible diphtheria toxin receptor mice (where MKs are depleted by inducible diphtheria toxin receptor expression) crossed with *Cxcl4*-cre mice, Bruns et al[16] showed that short-term (7 days) depletion of *Cxcl4* led to an increase in HSC proliferation, a 4.6-fold increase in HSC numbers, and enhanced reconstitution following a single transplantation. However, more extended *Cxcl4* depletion (for 6 weeks) resulted in an attenuated effect, and the authors suggested that the reduced quiescence driven by loss of *Cxcl4* may lead in time to HSC exhaustion as previously described.[4,48] We showed that complete loss of *Cxcl4* caused a decrease in HSC numbers, followed by a decrease in self-renewal capacity detected after secondary transplantation. It is therefore likely that *Cxcl4* inhibition does lead at first to a temporary increase in HSC numbers (seen by Bruns et al), and that this effect is then followed by HSC exhaustion and depletion (due to increased proliferation and differentiation) and by a decrease in self-renewal capacity as we observed in our serial transplantation assay.

By interrogating a previously published transcriptional array, we observed that in mice *Cxcl4* was the only chemokine ligand upregulated in the HSC compartment (unpublished observations and Månsson et al[31]), providing a rationale to investigate this factor in vivo. Our results indicated that mice lacking *Cxcl4* showed changes in LSK, MPP, and HSC numbers, and reduced secondary transplantation capacity, possibly due to changes in the microenvironment as well as in autocrine HSC signaling itself. Although several chemokines act through CXCR2, CXCL4 is not known to do so and thus this receptor does not provide a unifying explanation for the similarity in function of CXCL4 and CXCR2. One possibility is that CXCR2 ligands and CXCL4 function as a heterodimer within the BM, which mediates CXCR2-dependent signaling. Such a mechanism is supported by the clear evidence of complex heterodimeric interactions between chemokines, which lead to biological outcomes different from those of either of the individual component chemokines.[49-51]

Similar to what happens in other systems,[52-55] our results showed that the CXCR2 signaling pathway played an important role in HSC maintenance. CXCR2 inhibition with the specific CXCR2 inhibitor SB225002 replicated the results for knockdown of CXCL4. Importantly, despite previous studies having shown that human primitive HSCs do not express CXCR2, we were able to clearly detect CXCR2 protein expression in human CD34^{+}38^{-} and CD34^{+}38^{+} cells by immunofluorescence.[56,57] To further characterize the known effect on the myeloid compartment in *Cxcr2*$^{-/-}$ mice,[34] we investigated how its deletion affected the primitive HSC compartment. *Cxcr2*$^{-/-}$ mice exhibit a marked expansion of viable LSK, HSC, and HPC in the BM and spleen, whereas analysis of serial transplantation data showed significantly reduced long-term repopulating ability of these cells. Taken together, these results indicate that *Cxcr2* deficiency leads to a marked perturbation in normal hematopoietic homeostasis.

Our previous research has shown that differences in chemokine expression between quiescent and primitive cells is maintained in normal as well as in leukemic (chronic myeloid leukemia [CML]) HSCs.[23] Recently, it has been shown that CCL3 expression is required for the development of CML in mice, and that inflammatory chemokines such as CCL3, promote leukemia development.[21] Therefore, high levels of chemokine expression in primitive HSC/leukemic stem cells may suggest that chemokine signaling pathways play a role in the development and maintenance of hematologic cancers of stem cell origin. With Zhang et al, we have previously indicated that CML leukemic stem cells show a decreased homing ability due to a lower CXCL12 expression in CML BM compared with HSC, and treatment with the tyrosine kinase inhibitor imatinib reverted the abnormal cytokine levels and HSC growth.[20] Similarly, multipotent stromal cells derived from mouse models of myeloproliferative neoplasia have been shown to remodel the BM niche into a self-reinforcing leukemic niche, through expression of the chemokine CCL3.[22] In light of our novel findings presented here, although chemokines could represent a novel therapeutic target in

myeloproliferative disorders, prudence would be advised because some chemokine family members regulate survival and self-renewal in normal HSCs, and their modulation may be detrimental in the longer term.

Collectively, these data indicate that chemokines play an important role in human and mouse HSC survival, and maintenance. These studies represent a starting point for elucidation of the role of the chemokine family in hematopoietic homeostasis, both in normal stem cells and in leukemia; however, further investigations into the mechanisms through which the chemokines act at the level of HSCs are required to fully understand HSC behavior in the BM niche.

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Authorship

Contribution: A.S., L.P., F.P., and L.E.M.H. designed and performed research, analyzed data, and wrote the manuscript; M.S., K.D., and M.D. performed experiments and reviewed the manuscript; S.A.A., S.C., and A.V.G. assisted with some in vitro experiments; R.K., O.S., A.M.M., L.M., K.R.K., and G.J.G. provided material, interpreted data, and reviewed the manuscript; and T.L.H. designed the study, analyzed data, and wrote the manuscript.

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