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Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches

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Abstract

While haematopoietic stem cells (HSCs) are commonly assumed to reside within a specialized microenvironment, or niche[1], most published experimental manipulations of the HSC niche have also impacted the function of diverse restricted progenitors. This raises the fundamental question of whether HSCs[1] and restricted progenitors[2,3] reside within distinct, specialized niches or whether they share a common niche. Here we assess the physiological sources of the chemokine, CXCL12, for HSC and restricted progenitor maintenance. *Cxcl12*^{DsRed} knock-in mice showed that *Cxcl12* was primarily expressed by perivascular stromal cells and at lower levels by endothelial cells, osteoblasts, and some haematopoietic cells. Conditional deletion of *Cxcl12* from haematopoietic cells or *Nestin-cre*-expressing cells had little or no effect on HSCs or restricted progenitors. Deletion of *Cxcl12* from endothelial cells depleted HSCs but not myeloerythroid or lymphoid progenitors. Deletion of *Cxcl12* from perivascular stromal cells depleted HSCs and certain restricted progenitors and mobilized these cells into circulation. Deletion of *Cxcl12* from osteoblasts depleted certain early lymphoid progenitors, but not HSCs or myeloerythroid progenitors and did not mobilize these cells into circulation. Different stem/progenitor cells thus occupy distinct cellular niches in bone marrow: HSCs in a perivascular niche and early lymphoid progenitors in an endosteal niche.

Using SLAM family markers that isolate quiescent HSCs[4–8] we found that most HSCs localize adjacent to sinusoidal blood vessels in the bone marrow[4,9,10]. Using independent approaches others obtained similar results[11–13]. We therefore hypothesized that there is a perivascular niche for HSC maintenance[4]. Consistent with this, Stem Cell Factor (SCF) is primarily or exclusively expressed in the bone marrow by endothelial cells and perivascular stromal cells[10]. Conditional deletion of *Scf* from endothelial cells or *Leptin receptor* (*Lepr*)-expressing perivascular stromal cells depleted HSCs[10]. Combined deletion of *Scf* from both

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AUTHOR CONTRIBUTIONS

L.D. performed all of the experiments. L.D. and S.J.M. conceived the project, designed the experiments, interpreted the results, and wrote the manuscript.

AUTHOR INFORMATION

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endothelial cells and perivascular stromal cells caused severe HSC depletion and anemia. In contrast, conditional deletion of *Scf* from osteoblasts or haematopoietic cells did not affect HSC frequency or function. This proves there is a perivascular niche for HSC maintenance and raises the question of whether other haematopoietic progenitors reside in distinct niches.

CXCL12 is a chemokine required for HSC maintenance and retention in the bone marrow[11,14–17]. Global conditional deletion of *Cxcl12,* or the gene that encodes its receptor, *Cxcr4*, depletes HSCs from adult bone marrow[11,14]. CXCL12 also promotes the proliferation and maintenance of B lineage progenitors[15,18] and common lymphoid progenitors (CLPs)[19]. CXCL12 is expressed by perivascular stromal cells, endothelial cells and osteoblasts[11,20,21]. Some have proposed that the physiological source of CXCL12 for HSC maintenance is osteoblasts[14] while others have proposed perivascular stromal cells[11]; however, *Cxcl12* has not yet been conditionally deleted from any candidate niche cell. Thus, the cellular sources of CXCL12 for the maintenance of HSCs and lymphoid progenitors remain uncertain.

To systematically examine the *Cxcl12* expression pattern we generated $Cxcl12^{DsRed}$ knock-in mice by recombining *DsRed-Express2* (*DsRed*) into the endogenous *Cxcl12* locus (Supplementary Fig. 1a–c). *Cxcl12* was primarily expressed by cells surrounding sinusoids throughout the bone marrow, irrespective of proximity to the endosteum (Fig. 1a–c; Supplementary Fig. 1d). *Cxcl12*-DsRed expression overlapped with endothelial marker staining, suggesting that endothelial cells were one source of CXCL12 (Fig. 1a–c); however, perivascular stromal cells also appeared to produce CXCL12 (Fig. 1a–c).

The perivascular *Cxcl12* expression pattern was very similar to the *Scf* expression pattern[10]. In $Scf^{gfp/+}$; $Cxcl12^{DsRed/+}$ mice we found a strong overlap in *Cxcl12*-DsRed and *Scf*-GFP expression by perivascular cells throughout the bone marrow (Fig. 1d–f). By flow cytometry, virtually all *Scf*-GFP$^+$ stromal cells were positive for *Cxcl12*-DsRed (Fig. 1j) and virtually all *Cxcl12*-DsRed$^+$ stromal cells were positive for *Scf*-GFP (Fig. 1k). Perivascular HSC niche cells therefore produce both SCF and CXCL12.

To quantitate *Cxcl12* expression in perivascular stromal cells we sorted CD45/Ter119$^-$PDGFRα$^+$ mesenchymal stem/stromal cells from enzymatically dissociated bone marrow. The *Lepr*-Cre-expressing perivascular stromal cells that contribute to the HSC niche by secreting SCF are uniformly positive for PDGFRα[10] and virtually all CD45/Ter119$^-$PDGFRα$^+$ bone marrow cells express *Scf*-GFP (Supplementary Fig. 1e, f). Approximately 90% of CD45/Ter119$^-$PDGFRα$^+$ perivascular stromal cells expressed *Cxcl12*-DsRed (Fig. 1l). By flow cytometry, approximately 70% of VE-cadherin$^+$ endothelial cells and 0.5% of CD45/Ter119$^+$ haematopoietic cells in the bone marrow also expressed *Cxcl12*-DsRed (Fig. 1m, n and Supplementary Fig. 1g).

In contrast to *Scf*, which is not detectably expressed by osteoblasts[10], we did observe very low levels of *Cxcl12*-DsRed expression by bone-lining cells (Fig. 1g–i). Almost 70% of *Col2.3*-GFP$^+$ osteoblasts isolated from enzymatically digested bone from *Col2.3-GFP;* $Cxcl12^{DsRed/+}$ mice expressed low levels of *Cxcl12*-DsRed (Fig. 1o).

By quantitative reverse transcription polymerase chain reaction (qRT-PCR), EYFP$^+$ perivascular stromal cells from *Lepr-cre; loxpEYFP* mice expressed *Cxcl12* at ~15,000-fold the level observed in unfractionated bone marrow (Fig. 1p). VE-cadherin$^+$ endothelial cells, *Col2.3*-GFP$^+$ osteoblasts, and *Cxcl12*-DsRed$^+$ haematopoietic cells expressed significantly lower levels of *Cxcl12* at ~120 fold, ~13 fold, and ~3 fold the levels observed in bone marrow cells (Fig. 1p).

We generated a floxed allele of *Cxcl12* (*Cxcl12^{fl}*) that led to a frameshift upon recombination (Supplementary Fig. 2a–c). The floxed allele itself did not appear to have any phenotype as unrecombined *Cxcl12$^{fl/fl}$* mice were born and matured into adulthood in normal numbers with normal HSC frequency and haematopoiesis (Supplementary Fig. 2d–f). We recombined *Cxcl12^{fl}* in the germline with *CMV-cre* mice to generate a *Cxcl12$^-$* predicted null allele. *Cxcl12$^{+/-}$* mice were born in expected numbers (Supplementary Fig. 2g) with normal cellularity, B cell frequency, and HSC frequency in the bone marrow and spleen (Supplementary Fig. 2h–j). In contrast, *Cxcl12$^{-/-}$* progeny were not born alive (Supplementary Fig. 2g) consistent with the known perinatal lethal phenotype of *Cxcl12* deficient mice[15].

Global deletion of *Cxcl12* by administering tamoxifen to 8-week old adult *Ubc-creER; Cxcl12$^{fl/fl}$* mice significantly reduced white blood cell counts (Supplementary Fig. 4a), lymphocyte frequencies (Supplementary Fig. 4b), bone marrow cellularity (Supplementary Fig. 4c) and CD150$^+$CD48$^-$Lineage$^-$Sca1$^+$cKit$^+$ HSC[4] frequency (Supplementary Fig. 4d). Bone marrow cells from *Ubc-creER; Cxcl12$^{fl/fl}$* mice also gave significantly lower levels of donor cell reconstitution in all major haematopoietic lineages upon transplantation into irradiated mice (Supplementary Fig. 4e). Consistent with an independently targeted *Cxcl12^{fl}* allele[14], these results demonstrate CXCL12 promotes adult HSC maintenance and lymphopoiesis.

HSCs do not express *Cxcl12* by flow cytometry (Supplementary Fig. 4f). However, since some other haematopoietic cells expressed *Cxcl12*-DsRed (Fig. 1n, p), we conditionally deleted *Cxcl12* from all haematopoietic cells in *Vav1-cre; Cxcl12$^{fl/fl}$* mice. Recombination was highly efficient in *Vav1-cre; Cxcl12$^{fl/fl}$* HSCs (Supplementary Fig. 5a). Adult *Vav1-cre; Cxcl12$^{fl/fl}$* mice had normal cellularity, HSC frequency (Supplementary Fig. 4g, h), lineage composition in the bone marrow and spleen (Supplementary Fig. 5b), and reconstituting potential in irradiated mice (except for a modest decline in T cell reconstitution; Supplementary Fig. 4i). Cells from the bone marrow, spleen and blood of *Vav1-cre; Cxcl12$^{fl/fl}$* mice formed normal numbers of haematopoietic colonies in culture (Supplementary Fig. 5c). We were unable to detect HSCs in the blood of *Vav1-cre; Cxcl12$^{fl/fl}$* mice when we competitively transplanted 600,000 mononucleated blood cells into irradiated mice (data not shown).

Adult *Vav1-cre; Cxcl12$^{fl/fl}$* mice also had normal frequencies of CD150$^-$CD48$^-$LSK multipotent progenitors (MPPs; see Supplementary Figure 3 for flow cytometry gates and references for each cell population), Flt3$^+$LSK lymphoid-primed MPPs (LMPPs), CD34$^+$FcγR$^-$Lineage$^-$Sca1$^-$cKit$^+$ common myeloid progenitors (CMPs), Lineage$^-$Sca1^{low}cKit^{low}Flt3$^+$IL7Rα$^+$ common lymphoid progenitors (CLPs),

CD34$^-$FcγR$^-$Lineage$^-$Sca1$^-$cKit$^+$ megakaryocytic/erythroid progenitors (MEPs), and CD34$^+$FcγR$^+$Lineage$^-$Sca1$^-$cKit$^+$ granulocyte/macrophage progenitors (GMPs) (Supplementary Fig. 4j, k). Committed B lineage progenitors including B220$^+$sIgM$^-$CD43$^+$CD24$^-$ pre-pro B cells, B220$^+$sIgM$^-$CD43$^+$CD24$^+$ pro B cells, B220$^+$sIgM$^-$CD43$^-$ pre B cells, and B220$^+$sIgM$^-$ B cells were also similar to controls (Supplementary Fig. 4l). *Cxcl12* expression by haematopoietic cells is therefore not required for the maintenance or retention of HSCs or restricted progenitors in adult bone marrow.

Nestin-cre; Cxcl12$^{fl/fl}$ mice also had normal bone marrow and spleen cellularity, HSC frequency, and haematopoietic lineage composition (Supplementary Fig 6a, b). Bone marrow cells from *Nestin-cre; Cxcl12$^{fl/fl}$* mice were indistinguishable from control cells in their capacity to give long-term multilineage reconstitution of irradiated mice (Supplementary Fig. 6c). The bone marrow of *Nestin-cre; Cxcl12$^{fl/fl}$* mice had normal frequencies of MPPs, LMPPs, CMPs, CLPs, MEPs, GMPs, committed B lineage progenitors (Supplementary Fig. 6d–f). *Nestin-cre; Cxcl12$^{fl/fl}$* mice also had normal frequencies of colony-forming progenitors in the bone marrow, spleen, and blood (Supplementary Fig. 6g–i). We were unable to detect HSCs in the blood of these mice in competitive reconstitution assays (data not shown). *Nestin-cre* expressing cells are therefore not a physiologically important source of CXCL12 for the maintenance of HSCs or restricted progenitors in the bone marrow. While HSC niche cells do not express endogenous *Nestin*, *Nestin-cre*, or *Nestin-creER*, they do appear to express the *Nestin*-GFP transgene[10], consistent with previously published results[12].

Given that *Cxcl12* from haematopoietic cells was not required by HSCs or restricted progenitors (Supplementary Fig. 4g–l), *Tie2-cre; Cxcl12$^{fl/fl}$* mice allowed us to test whether *Cxcl12* from endothelial cells is physiologically important. Adult *Tie2-cre; Cxcl12$^{fl/fl}$* mice had normal bone marrow and spleen cellularity (Fig. 2a), blood cell counts, and lineage composition in the bone marrow and spleen (Supplementary Fig. 7a, b). However, the frequency of CD150$^+$CD48$^-$Lineage$^-$Sca1$^+$cKit$^+$ HSCs in the bone marrow of *Tie2-cre; Cxcl12$^{fl/fl}$* mice was significantly lower than in littermate controls (Fig. 2b). Bone marrow cells from *Tie2-cre; Cxcl12$^{fl/fl}$* mice also gave significantly lower levels of donor cell reconstitution in all major hematopoietic lineages upon transplantation into irradiated mice (Fig. 2c). Endothelial cells are therefore a physiologically important source of CXCL12 for HSC maintenance. This likely reflects a requirement for CXCL12 produced by postnatal bone marrow endothelial cells because *Cxcl12* deficient mice do not exhibit HSC depletion during fetal development, only in postnatal bone marrow[17,22]. HSCs from *Tie2-cre; Cxcl12$^{fl/fl}$* mice had normal frequencies of BrdU$^+$ and Annexin V$^+$ cells, suggesting that HSCs may be depleted in these mice through premature differentiation (Supplementary Fig. 7c, d).

Tie2-cre; Cxcl12$^{fl/fl}$ mice had normal frequencies of MPPs, LMPPs, CMPs, CLPs, MEPs, GMPs, and B lineage progenitors in the bone marrow (Fig. 2d–f) and colony-forming progenitors in the bone marrow, spleen, and blood (Fig. 2g–i). We were unable to detect HSCs in the blood of these mice in competitive reconstitution assays (data not shown). CXCL12 produced by endothelial cells is therefore not required for the retention of HSCs or most restricted progenitors in bone marrow.

We deleted *Cxcl12* from perivascular stromal cells in the bone marrow using *Lepr-cre*[10]. *Lepr-cre; Cxcl12$^{fl/-}$* mice had normal bone marrow and spleen cellularity (Fig. 3a), normal blood cell counts, and normal lineage composition in the bone marrow and spleen (Supplementary Fig. 7e, f). CD150$^+$CD48$^-$Lineage$^-$Sca1$^+$cKit$^+$ HSC frequency was normal in the bone marrow of *Lepr-cre; Cxcl12$^{fl/-}$* mice (Fig. 3b) and the reconstituting capacity of bone marrow cells in irradiated mice was normal (Fig. 3c). The bone marrow of *Lepr-cre; Cxcl12$^{fl/-}$* mice also had normal frequencies of MPPs, LMPPs, CMPs, CLPs, MEPs, GMPs, and B lineage progenitors (Fig 3d–f). However, the frequencies of HSCs and colony-forming progenitors were significantly elevated in the blood and spleen of *Lepr-cre; Cxcl12$^{fl/-}$* mice (Fig. 3b, h–j). This demonstrates that CXCL12 expression by *Lepr*$^+$ perivascular cells is required to retain HSCs and colony-forming progenitors in the bone marrow.

Col2.3-Cre recombines genes in fetal and adult osteoblasts[10,23]. *Col2.3-cre; Cxcl12$^{fl/fl}$* mice had normal bone marrow and spleen cellularity, normal CD150$^+$CD48$^-$Lineage$^-$Sca1$^+$cKit$^+$ HSC frequency in the bone marrow and spleen (Fig. 4a, b), normal blood cell counts, normal lineage composition in the bone marrow and spleen (Supplementary Fig. 8a, b). *Col2.3-cre; Cxcl12$^{fl/fl}$* bone marrow cells gave normal levels of donor myeloid reconstitution, but significantly lower levels of T and B cell reconstitution in irradiated mice relative to control cells (Fig. 4c). In contrast, purified HSCs from *Col2.3-cre; Cxcl12$^{fl/fl}$* mice gave similar levels of reconstitution in all lineages as control HSCs (Fig. 4d and Supplementary Fig. 8c). This suggests that the reduction in T and B cell reconstitution from the bone marrow of *Col2.3-cre; Cxcl12$^{fl/fl}$* mice reflected the depletion of early lymphoid progenitors.

The bone marrow of *Col2.3-cre; Cxcl12$^{fl/fl}$* mice had normal frequencies of MPPs, CMPs, MEPs, and GMPs (Fig. 4e) but significantly fewer CLPs and IL7Rα$^+$LMPPs – cells thought to be specified for lymphoid differentiation[24,25] (Fig. 4f, g). The thymus in *Col2.3-cre; Cxcl12$^{fl/fl}$* mice had a normal frequency of early thymic progenitors (B220$^-$Gr1$^-$Ter119$^-$CD4$^-$CD8$^-$CD44$^+$cKit$^+$CD25$^-$ ETPs; Supplementary Fig. 8d, e) and the bone marrow had a normal frequency of committed B lineage progenitors (Fig. 4h). This suggests that homeostatic mechanisms can restore normal frequencies of downstream lymphoid progenitors despite depletion of primitive lymphoid progenitors. CLPs from *Col2.3-cre; Cxcl12$^{fl/fl}$* mice had normal frequencies of BrdU$^+$ and Annexin V$^+$ cells, suggesting that these cells might be depleted by premature differentiation (Supplementary Fig. 8f, g).

Col2.3-cre; Cxcl12$^{fl/fl}$ mice had normal frequencies of myeloerythroid colony-forming progenitors in the bone marrow, spleen, and blood (Fig. 4i) and we were unable to detect HSCs in the blood of these mice in competitive reconstitution assays (data not shown). CXCL12 from osteoblasts is thus required for the maintenance of certain early lymphoid progenitors but not for the maintenance or bone marrow retention of HSCs or myeloerythroid progenitors.

In stained bone marrow sections we found 30% of Lineage$^-$IL7Rα$^+$ cells at the endosteum, adjacent to bone-lining cells (Fig. 4j and Supplementary Fig. 9). Only 5% of all bone marrow cells were adjacent to the endosteum in the sections we examined, so these early

lymphoid progenitors were 6 times more likely than the average bone marrow cell to be adjacent to endosteum. Both functional genetic data and localization data therefore suggest there is an endosteal niche for a subset of early lymphoid progenitors.

To test whether both HSCs and early lymphoid progenitors would be depleted by deleting *Cxcl12* in perivascular stromal cells and in osteoblasts we used *Prx1-cre*[26]. Greenbaum et al. discovered that *Prx1*-Cre deletes broadly in perivascular stromal cells as well as in osteoblasts[27]. Consistent with this, *Prx1*-Cre recombined a conditional reporter in osteoblasts as well as in 95±4% CD45/Ter119^{-}PDGFRα^{+} perivascular stromal cells (Supplementary Fig. 10a–c) but not in bone marrow endothelial cells (Supplementary Fig. 10d). Thus, *Prx1*-Cre recombined more broadly in perivascular stromal cells than *Lepr*-Cre, which recombined a conditional reporter in 70±9% of CD45/Ter119^{-}PDGFRα^{+} cells (Supplementary Fig. 1f). Consistent with our results with other Cre alleles, *Prx1-cre; Cxcl12$^{fl/fl}$* mice exhibited significant reductions in bone marrow cellularity (Fig. 4k) and in the frequencies of HSCs (Fig. 4l), CLPs, and IL7Rα^{+}LMPPs (Fig. 4m). The bone marrow of *Prx1-cre; Cxcl12$^{fl/fl}$* mice also had fewer committed B lineage progenitors, but CMPs, MEPs, and GMPs were normal (Supplementary Fig. 10e, f).

Our data indicate that HSCs depend upon a perivascular niche created by endothelial cells and *Lepr-cre*/*Prx1-cre*-expressing perivascular stromal cells, while some early lymphoid progenitors depend upon an endosteal niche created by osteoblasts, and committed B lineage progenitors depend upon a distinct perivascular niche created by *Prx1-cre*-expressing stromal cells but not endothelial cells. Greenbaum et al.[27] come to similar conclusions. Osteoblasts promote the proliferation and differentiation of lymphoid progenitors in culture and conditional ablation of osteoblasts in vivo acutely depletes lymphoid progenitors but not HSCs[28,29]. Conditional deletion of G protein alpha from osteoblasts depletes B lineage progenitors but not other haematopoietic progenitors[30]. Our data are thus consistent with a number of prior studies, including our recent work[10], in suggesting that osteoblasts create a niche for certain early lymphoid progenitors but not for HSCs.

SUPPLEMENTARY METHODS

Mice

Targeting vectors for making *Cxcl12*^{DsRed} and *Cxcl12*^{fl} mice were generated by recombineering[31]. Linearized targeting vector was electroporated into C57BL-derived Bruce4 ES cells. Correctly targeted clones were identified by Southern blotting. Following expansion, these ES cell clones were injected into C57BL/6-Tyr^{c-2J} blastocysts. Chimeric mice were bred with C57BL/6-Tyr^{c-2J} mice to obtain germline transmission. The Frt-flanked *Neo* selection cassette was removed by subsequent mating with *Flpe* mice[32]. The resulting mice were backcrossed for at least three generations onto a C57BL/Ka background. Mice used in this study included *Ubc-creER*[42], *CMV-cre*[43], *Vav1-cre*[44], *Nestin-cre*[45], *Tie2-cre*[46], *Lepr-cre*[47], *Prx1-cre*[26], and *LoxpEYFP*[48] (all from Jackson Laboratory). *Scf*$^{gfp/+}$ mice were described previously[10]. *Col2.3-cre* mice[23] were obtained from Drs. F. Liu and B. Kream (University of Connecticut). *Col2.3*-GFP mice[49] were obtained from Dr. D. Rowe. Tamoxifen chow (Harlan) containing tamoxifen citrate (Spectrum Chemical) at 400mg/kg,

with 5% sucrose added, was administrated to mice for 2–4 months to induce recombination by CreER.

All mice were housed in the Unit for Laboratory Animal Medicine at the University of Michigan or in the Animal Resource Center at the University of Texas Southwestern Medical Center. All protocols were approved by the University of Michigan Committee on the Use and Care of Animals and by the UT Southwestern Institutional Animal Care and Use Committee.

PCR genotyping

Primers for genotyping of the *Cxcl12*^{DsRed} allele were: OLD322, 5′-GATGCGCGTCAGAGACCCC-3′; OLD355, 5′-GCGCAGAGCTGCGAGCCTTTC-3′; and OLD 415, 5′-CTTGAAGCGCATGAAGGGCTTG-3′. Primers for genotyping of the *Cxcl12*^{fl} allele were: OLD312, 5′-GAGCCCAGAACTCTGCCACC-3′; and OLD313, 5′-TCTTGCAAAGACCATCCCCTC-3′. Primers for genotyping of the *Cxcl12*$^{-}$ allele were: OLD312, 5′-GAGCCCAGAACTCTGCCACC-3′, and OLD353, 5′-ACTCTGAGTGAGATATTCACATC-3′.

Long-term competitive reconstitution assay

Adult mice were administered a minimum lethal dose of radiation using a Cesium 137 GammaCell40 Irradiator (MDS) or an XRAD 320 x-ray irradiator (Precision X-Ray Inc.) to deliver two doses of 540 rads (total 1080 rads) at least 2 hours apart. C57BL/6-SJL (CD45.1) mice were used as recipient mice in transplantation experiments. Cells were injected into the retro-orbital venus sinus of anaesthetized mice. Blood cells were subjected to Ficoll centrifugation prior to transplantation to isolate mononucleated cells. Mononucleated cells were counted prior to transplantation either using a CBC machine or a hemocytometer with Turks solution. In most experiments recipient mice were maintained on antibiotic water (neomycin sulfate 1.11g/l and polymixin B 0.121g/l) for 14 days after transplantation and then switched to regular water. Recipient mice were bled from 4 to 16 weeks after transplantation to examine the levels of donor-derived myeloid, B and T cells in their blood. Red blood cells were lysed with ammonium chloride potassium buffer before antibody staining. The antibodies used to analyze donor chimerism in the blood were anti-CD45.1 (A20), anti-CD45.2 (104), anti-Gr1 (8C5), anti-Mac1 (M1/70), anti-B220 (6B2) and anti-CD3 (KT31.1).

Flow cytometry

Bone marrow cells were isolated by flushing the long bones or by crushing the long bones with mortal and pestle in Ca^{2+} and Mg^{2+} free HBSS with 2% heat-inactivated bovine serum. Spleen cells were obtained by crushing the spleen between two glass slides. The cells were dissociated to a single cell suspension by gently passing through a 25G needle then filtering through a 70μm nylon mesh. The following antibodies were used to isolate HSCs: anti-CD150 (TC15-12F12.2), anti-CD48 (HM48-1), anti-CD41 (MWReg30), anti-Sca1 (E13-161.7), anti-cKit (2B8) and the following antibodies against lineage markers (anti-Ter119, anti-B220 (6B2), anti-Gr1 (8C5), anti-CD2 (RM2-5), anti-CD3 (17A2), anti-CD5 (53-7.3) and anti-CD8 (53-6.7)). Haematopoietic progenitors were identified by flow

cytometry using the following antibodies: anti-Sca1 (E13-161.7), anti-cKit (2B8) and the following antibodies against lineage markers (anti-Ter119, anti-B220 (6B2), anti-Gr1 (8C5), anti-CD2 (RM2-5), anti-CD3 (17A2), anti-CD5 (53-7.3) and anti-CD8 (53-6.7)), anti-CD34 (RAM34), anti-CD135 (Flt3) (A2F10), anti-CD16/32 (FcγR) (93), anti-CD127 (IL7Rα) (A7R34), anti-CD24 (M1/69), anti-CD43 (1B11), anti-B220 (6B2), anti-IgM (II/41), anti-CD3 (17A2), anti-Gr1 (8C5), anti-Mac1 (M1/70), anti-CD41 (MWReg30), anti-CD71 (C2), anti-Ter119, anti-CD44 (IM7) and anti-CD25 (PC61). DAPI was used to exclude dead cells.

For flow cytometric analysis of *Scf*-GFP+ or *Lepr-cre; loxpEYFP*+ stromal cells, bone marrow was flushed using HBSS- with 2% bovine serum. Then whole bone marrow was digested with Collagenase IV (200U/ml) and DNase I (200U/ml) at 37°C for 15min. Samples were then stained with antibodies and analyzed by flow cytometry. Anti-CD140a (APA5), anti-CD45 (30F-11) and anti-Ter119 antibodies were used to isolate perivascular stromal cells. For analysis of bone marrow endothelial cells, mice were i.v. injected with 10ug/mouse Alexa Fluor 647 conjugated anti-VE-cadherin antibody (BV13, eBiosciences)[51]. Ten minutes later, the long bones were removed and bone marrow was flushed, digested and stained as above. Samples were run on FACSAria or FACSCanto II flow cytometers. Data were analyzed by FACSDiva (BD) or FlowJo (Tree Star) software. Osteoblasts were dissociated as described[50].

Methylcellulose cultures

Cells were sorted into methylcellulose culture medium (3434, Stemcell Technology) and incubated at 37°C as described[9]. Blood cells were obtained by Ficoll centrifuge according to manufacture's recommendation before plating (GE Healthcare).

Immunostaining bone sections

Freshly dissected long bones were fixed in a Formalin-based fixative at 4°C for 3 hours. Then the bones were embedded in 8% gelatin in PBS. Samples were snap frozen with liquid nitrogen and stored at −80°C. Bones were sectioned using CryoJane (Instrumedics). Sections were dried overnight at room temperature (RT) and stored at −80°C. Sections were re-hydrated in PBS for 5 min before immunostaining. 5% goat serum in PBS was used for blocking. Primary antibodies were applied to the slides for 1 hour at RT, followed by secondary antibody incubation for 30min at RT with repeated washes in between. Slides were mounted with anti-fade prolong gold (Invitrogen) and images were acquired on an Olympus IX81 microscope or a Zeiss LSM 780 confocal microscope. Primary antibodies were: chicken-anti-GFP (Aves), rat-anti-mouse pan-endothelial cell antigen (Meca32, Biolegend), rat-anti-endoglin (eBioscience), rat-anti-RFP (AlleleBiotechnology) and goat-anti-osteopontin (R&D). For staining $Lin^{-}IL7R\alpha^{+}$ cells in bone marrow sections, mice were intravenously injected with 2ug Alexa Fluor 647 conjugated monoclonal antibody against IL7Rα (A7R34, Biolegend). After 5 minutes, the mice were killed, and long bones were dissected, fixed, sectioned, and stained with antibodies against Lineage markers (CD2, CD3, CD5, CD8, Ter119, Gr1 and B220) as described above. Images were acquired using a Zeiss LSM 780 confocal microscope.

Quantitative reverse transcription PCR

Cells were sorted directly into Trizol. Total RNA was extracted according to manufacture's instructions. Total RNA was subjected to reverse transcription using SuperScript III (Invitrogen). Quantitative real-time PCR was run using SYBR green on a LightCycler 480 (Roche). β-actin was used to normalize the RNA content of samples. Primers used in this study were: *Cxcl12*: OLD35, 5′-TGCATCAGTGACGGTAAACCA-3′ and OLD36, 5′-GTTGTTCTTCAGCCGTGCAA-3′. β-actin: OLD27, 5′-GCTCTTTTCCAGCCTTCCTT-3′ and OLD28, 5′-CTTCTGCATCCTGTCAGCAA-3′.

Supplementary Material

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Acknowledgments

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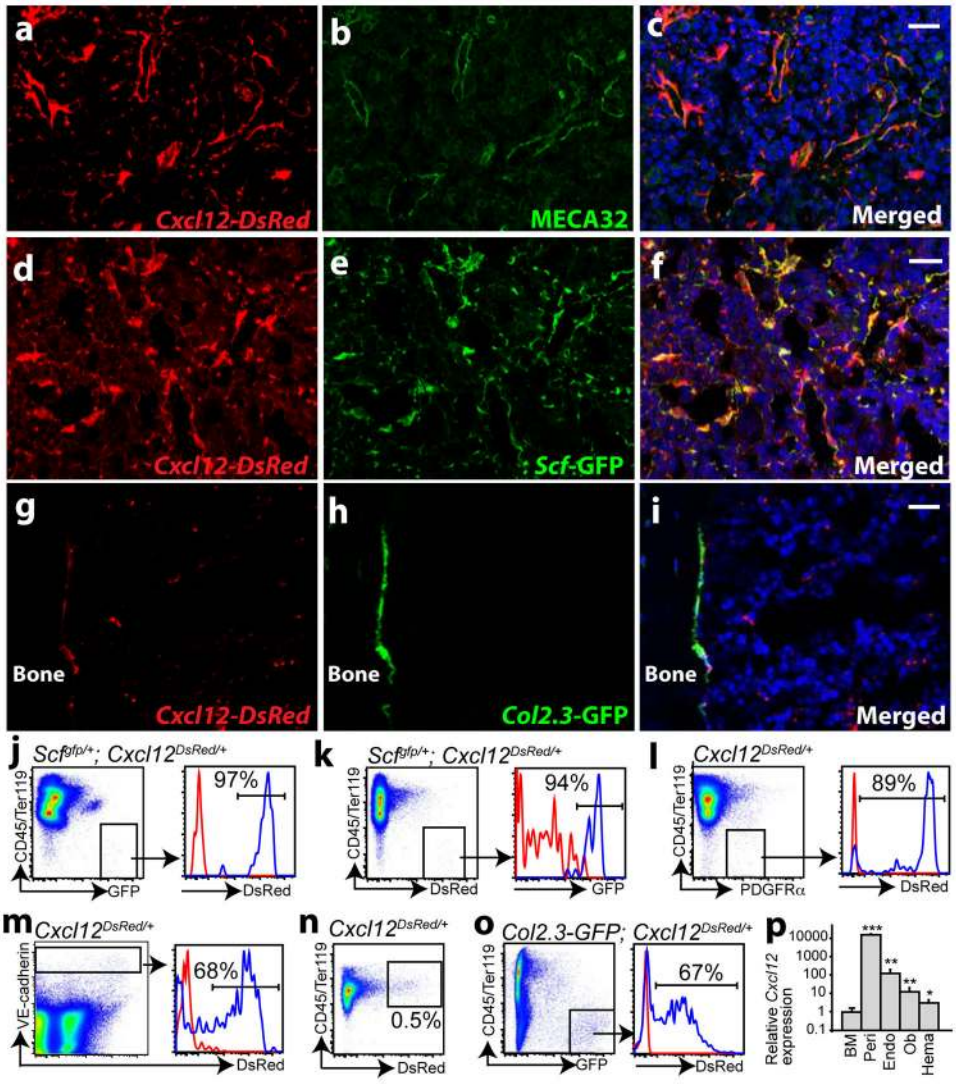

Figure 1. Endothelial cells and perivascular stromal cells are the major sources of *Cxcl12* in bone marrow

a–**c**, In *Cxcl12*$^{DsRed/+}$ mice, DsRed was primarily expressed by perivascular cells throughout the bone marrow. **d**–**f**, *Cxcl12*-DsRed and *Scf*-GFP expression strongly overlapped around sinusoids throughout the bone marrow of *Scf*$^{gfp/+}$*; Cxcl12*$^{DsRed/+}$ mice. **g**–**i**, *Col2.3*-GFP^{+} bone-lining osteoblasts expressed *Cxcl12*-DsRed in the bone marrow of *Col2.3-GFP*$^{+}$*; Cxcl12*$^{DsRed/+}$ mice. Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI, blue) in **c, f** and **i. j**, CD45/Ter119^{-}*Scf*-GFP^{+} perivascular stromal cells expressed *Cxcl12*-DsRed in *Scf*$^{gfp/+}$*; Cxcl12*$^{DsRed/+}$ (blue histogram) but not in *Scf*$^{gfp/+}$ control marrow (red histogram). **k**, CD45/Ter119^{-}*Cxcl12*-DsRed^{high} cells expressed *Scf*-GFP in *Scf*$^{gfp/+}$*; Cxcl12*$^{DsRed/+}$ (blue) but not in *Cxcl12*$^{DsRed/+}$ control marrow (red). **l**, CD45/Ter119^{-}PDGFRα^{+} perivascular stromal cells expressed *Cxcl12*-DsRed in *Cxcl12*$^{DsRed/+}$ (blue) but not control (+/+; red) marrow. **m**, VE-cadherin^{+} endothelial cells expressed *Cxcl12*-DsRed in *Cxcl12*$^{DsRed/+}$ (blue) but not control (+/+; red) marrow. **n**, 0.5% of CD45/Ter119^{+} haematopoietic cells expressed *Cxcl12*-DsRed in *Cxcl12*$^{DsRed/+}$ but not in

control marrow (Supplementary Fig. 1g). **o**, CD45/Ter119^{-}*Col2.3*-GFP^{+} osteoblasts from enzymatically dissociated bone expressed *Cxcl12*-DsRed in *Col2.3-GFP; Cxcl12*$^{DsRed/+}$ (blue) but not *Col2.3-GFP* control (red) mice. **p**, *Cxcl12* transcript levels by qRT-PCR in different subpopulations of bone marrow cells (n=3–6). BM, whole bone marrow cells. Peri, EYFP^{+} perivascular stromal cells from *Lepr-cre; loxpEYFP* mice. Endo, VE-cadherin^{+} bone marrow endothelial cells. Ob, *Col2.3*-GFP^{+} osteoblasts. Hema, CD45/Ter119^{+}*Cxcl12*-DsRed^{+} haematopoietic cells. All data represent mean±s.d. from at least three independent experiments. Two-tailed student's t-tests were always used to assess statistical significance (*P<0.05, **P<0.01, ***P<0.001). Scale bars are 20 um.

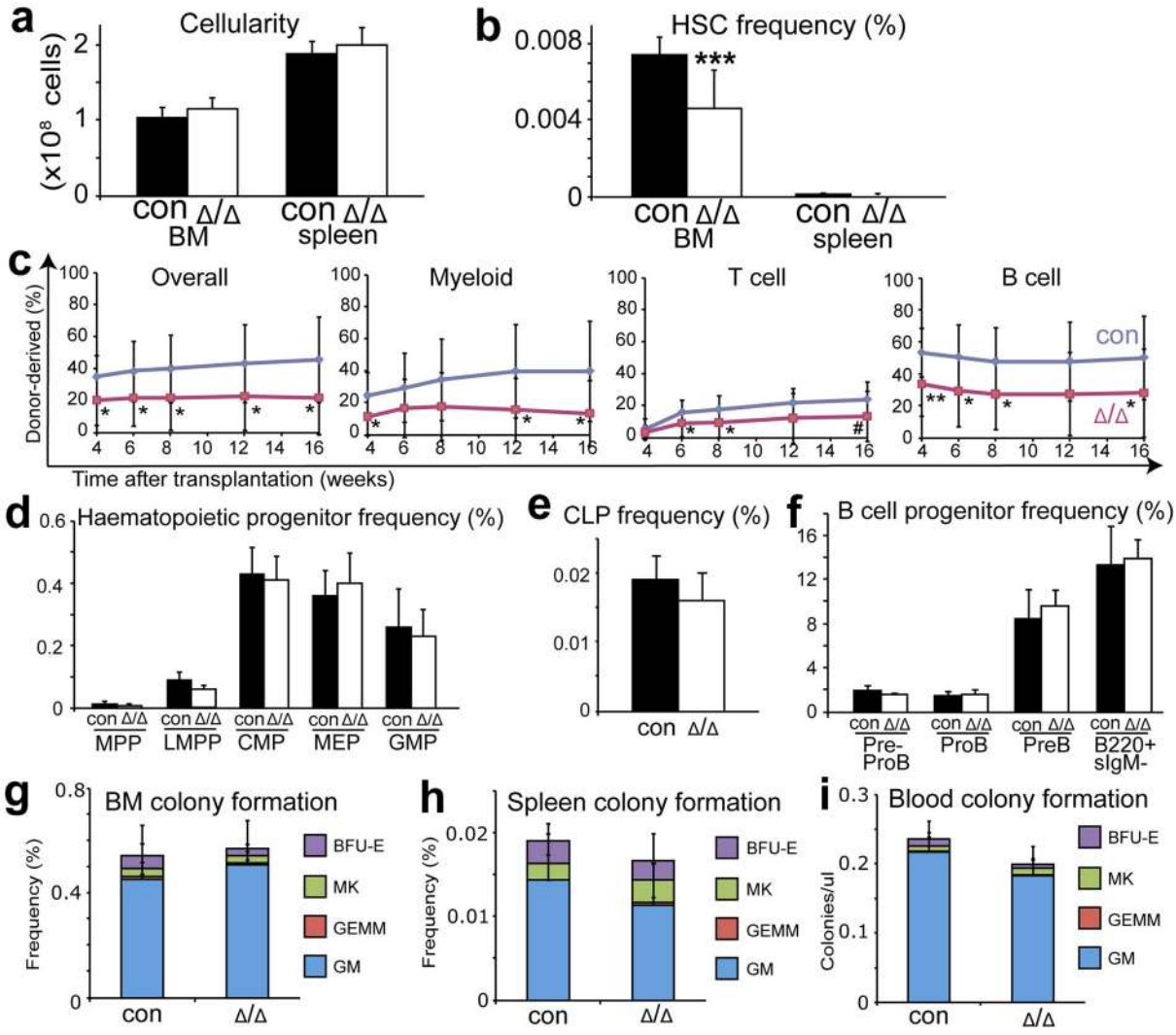

Figure 2. CXCL12 produced by endothelial cells promotes HSC maintenance

a, Bone marrow and spleen cellularity (n=8–9) and (**b**) HSC frequency in *Tie2-cre; Cxcl12$^{fl/fl}$* mice versus littermate controls (n=10–11). **c**, 3×10^5 donor bone marrow cells from *Tie2-cre; Cxcl12$^{fl/fl}$* mice gave significantly lower levels of donor myeloid, B, and T cell reconstitution in irradiated mice (three experiments with a total of 12–14 recipients per genotype). **d–f,** *Tie2-cre; Cxcl12$^{fl/fl}$* mice had normal frequencies of MPPs, LMPPs, CMPs, MEPs, GMPs (**d**), CLPs (**e**), and committed B lineage progenitors in bone marrow (**f**) (n=3–4). **g–i,** *Tie2-cre; Cxcl12$^{fl/fl}$* mice had normal frequencies of myeloerythroid colony-forming progenitors in bone marrow (**g**), spleen (**h**), and blood (**i**) (n=3–6). Δ, recombined *Cxcl12^{fl}* allele; con, negative control mice with +/+ or fl/+ or fl/fl *Cxcl12* genotypes (without *cre*). Data are mean±s.d. (*P<0.05, **P<0.01, ***P<0.001, #=0.057).

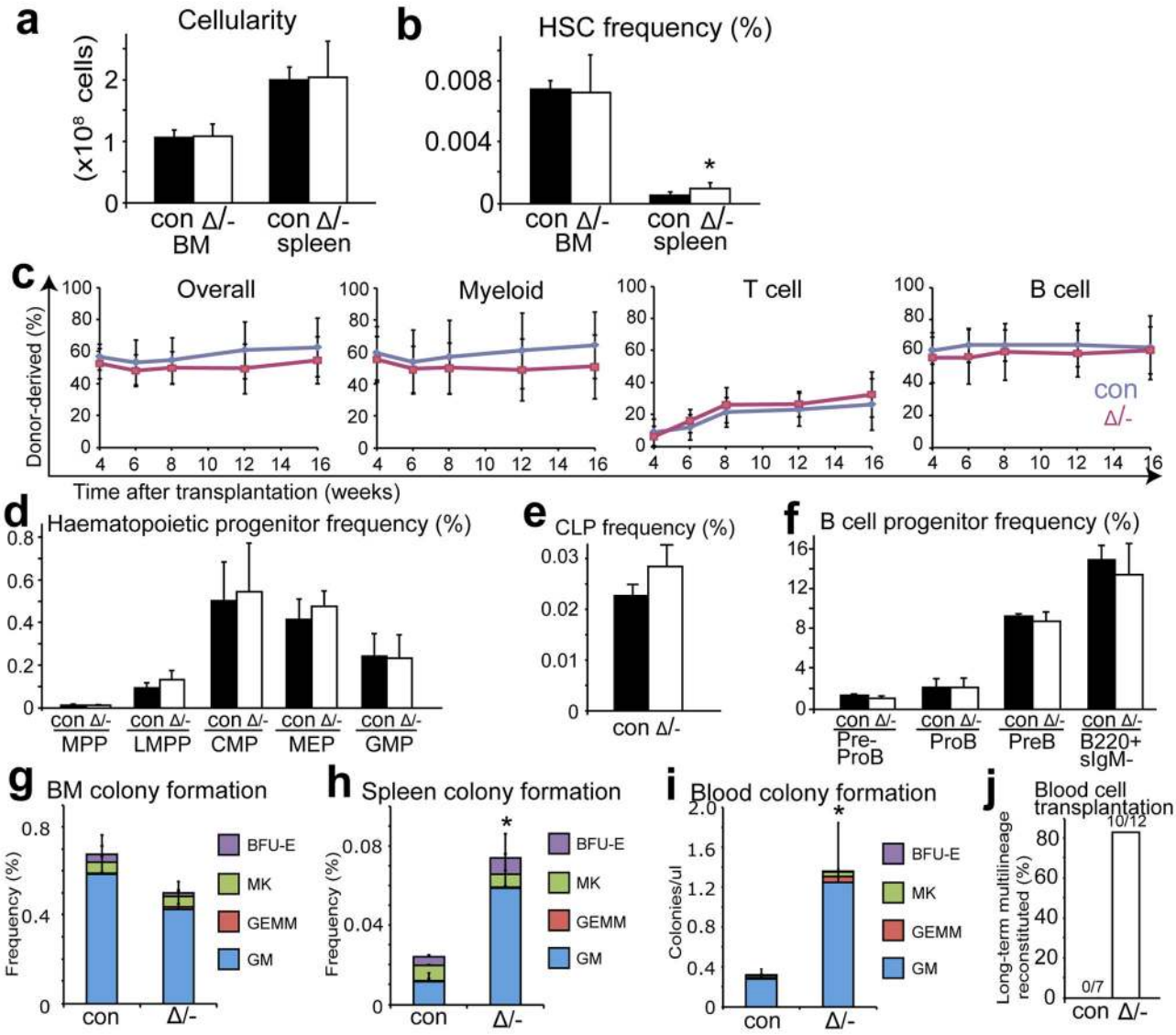

Figure 3. CXCL12 produced by *Lepr*-expressing perivascular stromal cells retains HSCs and colony-forming progenitors in the bone marrow

a, b, Cellularity (**a**, n=6) and HSC frequency (**b**, n=6–7) in the bone marrow and spleen of *Lepr-cre; Cxcl12$^{fl/-}$* mice and littermate controls. **c**, 3×10^5 bone marrow cells from *Lepr-cre; Cxcl12$^{fl/-}$* mice gave normal levels of donor myeloid, B, and T cell reconstitution in irradiated mice (three experiments with a total of 15 recipient mice per genotype). **d–f**, *Lepr-cre; Cxcl12$^{fl/-}$* mice had normal frequencies of MPPs, LMPPs, CMPs, MEPs, GMPs (**d**), CLPs (**e**), and committed B lineage progenitors in bone marrow (**f**) (n=3). **g–i**, *Lepr-cre; Cxcl12$^{fl/-}$* mice had normal frequencies of myeloerythroid colony-forming progenitors in bone marrow (**g**) but significantly increased frequencies in spleen (**h**), and blood (**i**) (n=3–5). **j**, 6×10^5 mononucleated blood cells from *Lepr-cre; Cxcl12$^{fl/-}$* mice gave long-term multilineage reconstitution in irradiated mice, while blood cells from littermate controls did not. Δ, recombined *Cxcl12^{fl}* allele; -, germline deleted *Cxcl12* allele or *Cxcl12^{DsRed}* allele; con, control mice. Data represent mean±s.d. (*P<0.05, **P<0.01, ***P<0.001).

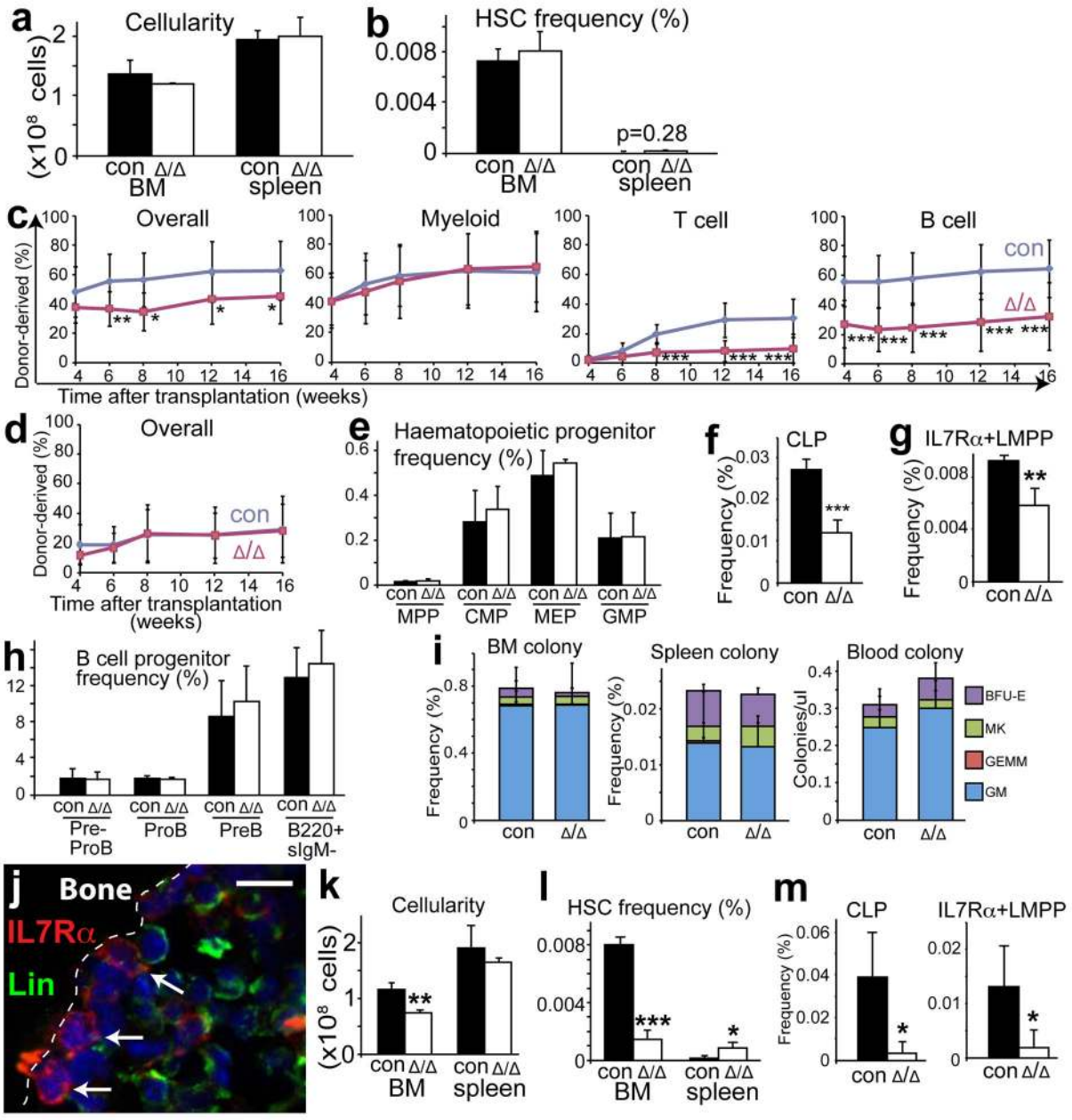

Figure 4. CXCL12 produced by osteoblasts promotes the maintenance of early lymphoid progenitors but not HSCs

a, b, Cellularity (**a**, n=4) and HSC frequency (**b**, n=4) in the bone marrow and spleen of *Col2.3-cre; Cxcl12$^{fl/fl}$* mice and littermate controls. **c**, 3×10^5 bone marrow cells from *Col2.3-cre; Cxcl12$^{fl/fl}$* mice gave significantly lower levels of donor cell reconstitution in the T and B cell lineages but not in the myeloid lineage relative to control bone marrow cells (three experiments with a total of 13–14 recipients per genotype). **d**, 20 CD150$^+$CD48$^-$Lineage$^-$Sca1$^+$cKit$^+$ HSCs from *Col2.3-cre; Cxcl12$^{fl/fl}$* mice gave normal donor cell reconstitution (three experiments with a total of 14–15 recipients per genotype), including normal levels of myeloid, B, and T cells (Supplementary Fig. 8c). **e–h,** *Col2.3-*

cre; Cxcl12$^{fl/fl}$ bone marrow had normal frequencies of MPPs, CMPs, MEPs, GMPs (**e**), and committed B lineage progenitors (**h**) but significantly reduced frequencies of CLPs (**f**) and IL7Rα^{+}LMPPs (**g**) (n=3–5). **i**, *Col2.3-cre; Cxcl12*$^{fl/fl}$ mice had normal frequencies of myeloerythroid colony-forming progenitors in the bone marrow, spleen, and blood (n=3–6). **j**, Some Lin^{-}IL7Rα^{+} early lymphoid progenitors were adjacent to the endosteum. **k–m**, *Prx1-cre; Cxcl12*$^{fl/fl}$ mice exhibited significant reductions in bone marrow cellularity (**k**, n=3–4) and the frequencies of HSCs (**l**), CLPs, and IL7Rα^{+}LMPPs (**m**, n=4–5).Δ, recombined *Cxcl12*^{fl} allele; con, control mice. *P<0.05, **P<0.01, ***P<0.001.