A perisinusoidal niche for extramedullary haematopoiesis in the spleen

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Haematopoietic stresses mobilize haematopoietic stem cells (HSCs) from the bone marrow to the spleen and induce extramedullary haematopoiesis (EMH). However, the cellular nature of the EMH niche is unknown. Here we assessed the sources of the key niche factors, SCF (also known as KITL) and CXCL12, in the mouse spleen after EMH induction by myeloablation, blood loss, or pregnancy. In each case, SCF was expressed by endothelial cells and Tcf21+ stromal cells, primarily around sinusoids in the red pulp, while CXCL12 was expressed by a subset of Tcf21+ stromal cells. EMH induction markedly expanded the SCF-expressing endothelial cells and stromal cells by inducing proliferation. Most splenic HSCs were adjacent to Tcf21+ stromal cells in red pulp. Conditional deletion of SCF from spleen endothelial cells, or of SCF or CXCL12 from Tcf21+ stromal cells, severely reduced spleen EMH and reduced blood cell counts without affecting bone marrow haematopoiesis. Endothelial cells and Tcf21+ stromal cells thus create a perisinusoidal EMH niche in the spleen, which is necessary for the physiological response to diverse haematopoietic stresses.
**Tcf21** + perisinoidal stromal cells express Scf

To identify *cre* alleles that recombine in spleen, but not bone marrow, stromal cells, we assessed the gene expression profile of spleen Scf-GFP+VE-cadherin− stromal cells (Extended Data Table 1). After testing a number of *cre* alleles (see Extended Data Fig. 2), we found that Tcf21-Cre/ER (ref. 21) recombinantly efficient in spleen Scf-GFP+ stromal cells (Fig. 2a) but not in bone marrow (Fig. 2b, c). Tcf21-Ires/ER, R26dTomato mice gavaged with tamoxifen for 12 days at 4–6 weeks of age expressed Tomato in Scf-GFP+ stromal cells throughout the red pulp (Fig. 2a, d), whereas Tomato was expressed only in rare white pulp cells (Fig. 2a) and in no endothelial cells (Fig. 2d, e). Tomato+/CD45−Ter119− stromal cells from enzymatically dissociated Tcf21-Ires/ER, R26dTomato spleens accounted for 0.085 ± 0.045% of spleen cells and 69 ± 2% of spleen CFU-Fs (Fig. 2f, g). These cells were PDGFR−β+ and LepR− (Fig. 2f).

In the liver, Scf-GFP was exclusively expressed by VE-cadherin+ endothelial cells (Extended Data Fig. 2a, b). Tcf21-Cre/ER recombinated in 0.09% of liver cells, none of which expressed Scf-GFP (Extended Data Fig. 2a, c). The Tcf21-Cre/ER recombination pattern did not significantly change in the spleen (Fig. 2f and Extended Data Fig. 2d, e), bone marrow (Extended Data Fig. 2f, g), or liver (Extended Data Fig. 2h, i) upon EMH induction by Cy−+21 d G-CSF.

c-Kit+ haematopoietic progenitors were almost exclusively within the red pulp in the normal spleen (Extended Data Fig. 3a, b) and after EMH induction (Fig. 2k). To assess HSC localization we used a new technique that permits deep imaging of α-catulin-GFP+c-Kit+ HSCs in optically cleared haematopoietic tissues22. In the spleens of mice treated with Cy+4 d G-CSF, only 0.019 ± 0.01% of splenocytes were α-catulin-GFP+c-Kit+ (Fig. 2h). All long-term multilineage reconstituting cells in the spleen were α-catulin-GFP+ and 28% of α-catulin-GFP−c-Kit+ spleen cells gave long-term multilineage reconstitution in primary (Fig. 2i) and secondary irradiated recipient mice (data not shown).

After antibody staining of a large segment of Tcf21-Ires/ER, R26dTomato, α-catulin-GFP spleen, we cleared the tissue (Extended Data Fig. 3c, d), then imaged to a depth of 300 μm and digitally reconstructed the tissue (Extended Data Fig. 3e, f and Supplementary Video 1). α-Catulin−GFP−c-Kit+ HSCs were found exclusively within the red pulp, whereas 80% were within 5 μm of Tomato+ stromal cells (Fig. 2j).

**EMH requires SCF and CXCL12 from Tcf21+ cells**

To test whether Tcf21-Ires/ER, expressing perivascular cells promote EMH, we treated 4–6-week-old Tcf21-Ires/ER, Scffl/fl and littermate control mice with tamoxifen for 12 days. A month later, bone marrow and spleen cellularity, blood cell counts, and bone marrow haematopoiesis were similar in Tcf21-Ires/ER, Scf−/− mice and littermates (Fig. 3a–f and Extended Data Fig. 3g–l). Then we treated Tcf21-Ires/ER, Scf−/− mice and littermates with cyclophosphamide followed by 4, 8, or 21 days of G-CSF. Tcf21-Ires/ER, Scf−/− mice did not differ from controls with respect to bone marrow cellularity (Fig. 3a) or the numbers of HSCs (Fig. 3b), common myeloid progenitors (CMPs23), granulocyte–macrophage progenitors (GMPs23), or megakaryocyte–erythroid progenitors (MEPs24) in the bone marrow after Cy+4–21 d G-CSF treatment (Extended Data Fig. 3j–l). In contrast, Tcf21-Ires/ER, Scf−/− mice had significantly fewer splenocytes (Fig. 3c), spleen HSCs (Fig. 3d), CMPs (Fig. 3e), GMPs (Extended Data Fig. 3m) and CMPs (Fig. 3f) relative to littermate controls after Cy−+8–21 d G-CSF treatment. We did not detect any difference between Tcf21-Ires/ER, Scf−/− mice and littermate controls in terms of vascular or stromal cell morphology in the spleen, with or without induction of EMH (Extended Data Fig. 4a–g). Conditional deletion of Scf with Tcf21-Cre/ER thus depletes HSCs and reduces EMH in the spleen without affecting bone marrow haematopoiesis.

Red blood cell (RBC) and white blood cell (WBC) counts were significantly lower in Tcf21-Ires/ER, Scf−/− mice as compared to controls after Cy−+8–21 d G-CSF treatment (Extended Data Fig. 3g–i). Splenocytes significantly reduced RBC and WBC counts in mice treated with Cy+G-CSF, demonstrating that splenic EMH is necessary for the
BM cellularity

85%

Tcf21cre/ER; R26tdTomato

††

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spleen cellularity (Fig. 3k) and numbers of spleen CMPs, GMPs and

and after Cy

the regeneration of blood cells after Cy

expression by

but negative for LepR, irrespective of EMH induction by Cy

Figure 2 | During EMH most HSCs localize adjacent to Tcf21+ stromal cells in the red pulp. a, Tamoxifen-treated adult Tcf21cre/ER; R26tdTomato mice exhibited widespread Tomato expression by perivascular stromal cells in the red pulp (RP). b, c, No Tomato expression in bone marrow from tamoxifen-treated Tcf21cre/ER; R26tdTomato mice. d, e, Most Scf-GFP+ VE-cadherin+ stromal cells were Tomato+ (arrows) whereas Scf-GFP+ VE-cadherin− endothelial cells were Tomato− (arrowhead). f, Tomato+ CD45− stromal cells from enzymatically dissociated spleen from Tcf21cre/ER; R26tdTomato mice were positive for PDGFR-β but negative for LepR, irrespective of EMH induction by Cy+ G-CSF. g, Percentage of all CFU-F colonies formed by enzymatically dissociated Tcf21cre/ER; R26tdTomato+ spleen cells that were Tomato+. Macrophage colonies were excluded by staining with anti-CD45 antibody. h, α-Catulin-GFP+ c-Kit+ HSCs represented 0.019 ± 0.01% of dissociated spleen cells in α-catulinGFP+ mice with EMH. i, α-Catulin-GFP+ c-Kit+ spleenocytes were highly enriched for long-term multilineage reconstituting (LTRM) HSCs. j, k, Deep imaging of α-catulin−GFP+ c-Kit+ HSCs (arrows in k) in optically cleared spleen from a Tcf21cre/ER; R26tdTomato+ c-Kit+ GFP− mouse with EMH induced by Cy+21 d G-CSF. The distance from α-catulin−GFP− c-Kit+ HSCs or random spots to Tomato+ stromal cells (j; *P < 0.05 by two-tailed Student’s t-test) was α-catulin−GFP− c-Kit+ HSCs were exclusively in the red pulp (k; see Extended Data Fig. 3f for a low-magnification view). All data reflect mean ± s.d. from 3 mice in 3 independent experiments.

recovery of blood cell counts (Fig. 3g, h and Extended Data Fig. 3n). However, conditional deletion of Scf by Tcf21-Cre/ER did not further reduce blood cell counts in splenectomized mice (Fig. 3g, h). SCF expression by Tcf21+ stromal cells in the spleen is thus necessary for the regeneration of blood cells after Cy+ G-CSF treatment.

Bone marrow cellularity and bone marrow haematopoiesis were similar in Tcf21cre/ER; Cxcl12fl/fl mice and littermate controls, before and after Cy+ G-CSF treatment (Fig. 3i, j and Extended Data Fig. 3r–t). However, Tcf21cre/ER; Cxcl12fl/fl mice exhibited significantly reduced spleen cellularity (Fig. 3k) and numbers of spleen CMPs, GMPs and MEPS (Fig. 3m, n and Extended Data Fig. 3u) relative to controls after Cy+8–21 d G-CSF treatment. Although the number of bone marrow (BM) cells (a) and bone marrow CD150+CD48+ LSK HSCs (b) in one femur plus one tibia as well as spleen (SP) cellularity (c) and the numbers of HSCs (d), CMPs (e) and MEPS (f) in the spleen. g, h, Sham-operated and splenectomized mice were treated with Cy+21 d G-CSF 1 month after surgery: WBC (g) and RBC (h) counts are shown. i–o, Tcf21cre/ER; Cxcl12fl/fl and Cxcl12fl/fl− or Cxcl12fl/fl− control mice were treated with tamoxifen then examined 1 month later either under normal conditions (not treated (NT)) or after treatment with Cy+4–21 d G-CSF to induce EMH. The number of bone marrow (BM) cells (a) and bone marrow CD150+CD48+ LSK HSCs (b) in one femur plus one tibia as well as spleen (SP) cellularity (c) and the numbers of HSCs (d), CMPs (e) and MEPS (f) in the spleen. g, h, Sham-operated and splenectomized mice were treated with Cy+21 d G-CSF 1 month after surgery: WBC (g) and RBC (h) counts are shown. i–o, Tcf21cre/ER; Cxcl12fl/fl and Cxcl12fl/fl− or Cxcl12fl/fl− control mice were treated with tamoxifen then examined 1 month later either under normal conditions (NT) or after treatment with Cy+4–21 d G-CSF to induce EMH. The number of bone marrow cells (i) and bone marrow HSCs (j) in one femur plus one tibia as well as spleen cellularity (k), numbers of HSCs (l), CMPs (m) and MEPS (n) in the spleen are shown. o, Number of HSCs per ml of blood in tamoxifen-treated control and Tcf21cre/ER; Cxcl12fl/fl mice after Cy+21 d G-CSF. The numbers of mice per treatment are shown in each bar in each panel. All panels reflect mean ± s.d. from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, statistical significance relative to sham-operated Scffl/fl mice. ††P < 0.05, †††P < 0.001, statistical significance among other treatments.

MEPs (Fig. 3m, n and Extended Data Fig. 3u) relative to controls after Cy+8–21 d G-CSF treatment. Although the number of HSCs in the spleens of Tcf21cre/ER; Cxcl12fl/fl− mice did not significantly differ from littermate controls (Fig. 3i), HSC numbers were significantly elevated in the blood (Fig. 3o) and in the bone marrow (Fig. 3i) of Tcf21cre/ER; Cxcl12fl/fl− mice after Cy+21 d G-CSF treatment. This suggests that some HSCs were mobilized from the spleens of Tcf21cre/ER; Cxcl12fl/fl− mice. Tcf21cre/ER; Cxcl12fl/fl− mice also had significantly reduced RBC counts after Cy+21 d G-CSF treatment (Extended Data Fig. 3o–q). We did not detect any difference between Tcf21cre/ER; Cxcl12fl/fl− mice and littermate controls in terms of the frequency or morphology of vascular or stromal cells in the spleen, with or without EMH.
EMH requires SCF from endothelial cells

We discovered that Vav1-Cre recombines efficiently in spleen, but not bone marrow, endothelial cells. Vav1-cre; R26<sup>dfTomato</sup>; Scf<sup>fl/fl</sup> mice recombined throughout the red pulp in VE-cadherin<sup>−</sup>Scf-GFP<sup>+</sup> cells but only in rare white pulp cells (Fig. 4a–c). VE-cadherin<sup>−</sup>Scf-GFP<sup>+</sup> cells accounted for 0.37 ± 0.07% of enzymatically dissociated spleen cells and 83 ± 5.3% of these cells recombined with Vav1-Cre (Fig. 4b). These cells were negative for PDGFR-β (Extended Data Fig. 5a). Seventy ± 5% of VE-cadherin<sup>−</sup> endothelial cells were Tomato<sup>+</sup> in the spleens of Vav1-cre; R26<sup>dfTomato</sup> mice but only 8.4 ± 0.5% were Tomato<sup>+</sup> in bone marrow (Extended Data Fig. 5b, e–h). Endothelial cells from Vav1-cre; Scf<sup>fl/fl</sup> mice exhibited a 6.5-fold reduction in Scf<sup>+</sup> transcript levels (Extended Data Fig. 5c) and a 5.6-fold reduction in SCF protein (Extended Data Fig. 5d) relative to endothelial cells from Scf<sup>fl/fl</sup>-controls.

In the livers of Vav1-cre; R26<sup>dfTomato</sup>; Scf<sup>fl/fl</sup> mice recombination occurred in 26 ± 4.2% of VE-cadherin<sup>−</sup>Scf-GFP<sup>+</sup> cells (Extended Data Fig. 5i–k). Upon induction of EMH by Cy<sup>+</sup>-G-CSF, Vav1-Cre recombination did not significantly change in the spleen (Extended Data Figs. 5b and 6a, b), bone marrow (Extended Data Figs. 5b and 6c, d) or liver (Extended Data Fig. 6e, f).

Cxc112 was not expressed by spleen endothelial cells (Fig. 1e). Consistent with this, Vav1-cre; Cxc112<sup>fl/fl</sup> mice had normal blood counts, cellularity, and numbers of HSCs, CMPs, GMPs and MEPs in bone marrow and spleen after Cy<sup>+</sup>-G-CSF (Extended Data Fig. 6g–i).

Vav1-Cre also recombines in haematopoietic cells<sup>24</sup> but haematopoietic cells do not express Scf and Vav1-cre; Scf<sup>fl/fl</sup> mice have normal HSC frequency and haematopoiesis in bone marrow<sup>18,19</sup>. Prior to EMH induction with Cy<sup>+</sup>-G-CSF, Vav1-cre; Scf<sup>fl/fl</sup> mice did not significantly differ from Scf<sup>−/−</sup>-controls with respect to bone marrow or spleen cellularity, or the numbers of HSCs, CMPs, GMPs or MEPs in the bone marrow or spleen (Fig. 4d–i and Extended Data Fig. 6w–z). After Cy<sup>+</sup>-G-CSF treatment, bone marrow cellularity and numbers of bone marrow HSCs, CMPs, GMPs or MEPs in Vav1-cre; Scf<sup>fl/fl</sup> mice were normal (Fig. 4d, e and Extended Data Fig. 6w–y). However, RBC counts, spleen cellularity, and the numbers of spleen HSCs, CMPs and MEPs declined in Vav1-cre; Scf<sup>fl/fl</sup> mice relative to Scf<sup>−/−</sup>-controls (Fig. 4f–i and Extended Data Fig. 6t–v).

The decline in blood cell counts in Vav1-cre; Scf<sup>fl/fl</sup>-mice after EMH induction was caused by reduced spleen EMH because splenectomy significantly reduced RBC and WBC counts but conditional deletion of Scf in splenectomized Vav1-cre; Scf<sup>fl/fl</sup>-mice had no further effect on blood cell counts (Fig. 4j, k). We did not detect any difference between Vav1-cre; Scf<sup>−/−</sup>-mice and controls in terms of the frequency or morphology of vascular or stromal cells in the spleen (Extended Data Fig. 4o–u). Endothelial SCF expression is thus necessary for splenic EMH and the recovery of blood cell counts after Cy<sup>+</sup>-G-CSF.

The splenic EMH niche during pregnancy

Erythropoiesis and myelopoiesis significantly increased in the red pulp (RP) during pregnancy, profoundly increasing spleen cellularity, HSC number, and progenitor numbers relative to non-pregnant mice (Extended Data Fig. 7a–i). Just as in Cy<sup>+</sup>-G-CSF-treated mice, Scf-GFP was largely expressed by endothelial and perivascular stromal cells in the red pulp and Cxcl12-DsRed was expressed by a subset of the Scf-GFP<sup>+</sup> stromal cells (Extended Data Fig. 7j–l). Pregnancy induced these cells to proliferate, significantly expanding their numbers (Extended Data Fig. 7m–o). In pregnant mice, Tcf21<sup>Cre/ER</sup> recombined in spleen PDGFRα<sup>−/−</sup>LepR<sup>+</sup> stromal cells but not in bone marrow and rarely in liver (Extended Data Fig. 7p–v). Vav1-cre; Scf<sup>−/−</sup>-mice were infertile, preventing us from testing the endothelial contribution to EMH during pregnancy.

Pregnant Tcf21<sup>Cre/ER</sup>, Scf<sup>−/−</sup> females did not differ from Scf<sup>−/−</sup>-control females in terms of bone marrow cellularity (Fig. 5a), or the numbers of HSCs (Fig. 5b), GMPs, CMPs or MEPs in the bone marrow (Extended Data Fig. 8a–d). In contrast, pregnant Tcf21<sup>Cre/ER</sup>, Scf<sup>−/−</sup> females exhibited significantly lower spleen cellularity and numbers of HSCs, GMPs, CMPs, MEPs, myeloid and erythroid cells in the spleen as compared to pregnant Scf<sup>−/−</sup>-females (Fig. 5c–f and Extended Data Fig. 8e, f). Pregnant Tcf21<sup>Cre/ER</sup>, Scf<sup>−/−</sup> females had significantly lower RBC counts than pregnant Scf<sup>−/−</sup>-controls (Fig. 5g), and significantly lower fetal mass (Fig. 5h). SCF from Tcf21<sup>−/−</sup> perivascular cells is thus necessary for splenic EMH and for the expansion of erythropoiesis during pregnancy.

Figure 4 | Endothelial cells are an important source of SCF for EMH in the spleen. a. Vav1-cre; R26<sup>dfTomato</sup> mice exhibited vascular Tomato expression throughout the splenic red pulp (RP). b. Tomato fluorescence in endothelial cells. WP, white pulp.

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Figure 5 | SCF from endothelial cells and Tcf21+ stromal cells is necessary for splenic EMH and adequate erythropoiesis after bleeding or during pregnancy. a–h, Four-to-six-month-old female mice that had been treated with tamofoxifen at least 2 months earlier were mated with normal wild-type males. a–f, Normal females and pregnant females at gestation day 18.5 were analysed: the number of bone marrow (BM) cells (a) and bone marrow HSCs (b) in one femur plus one tibia as well as spleen (SP) cellularity (c), and the numbers of HSCs (d), CMPs (e) and MEPs (f) in the spleen are shown. g, h, RBC counts (g) and fetal mass (h). i–n, Four-to-six-month-old mice with the indicated genetic backgrounds were repeatedly bled over a 2-week period then analysed: the number of bone marrow cells (i) and bone marrow HSCs (j) in one femur plus one tibia as well as spleen cellularity (k), and the numbers of HSCs (l), CMPs (m) and MEPs (n) in the spleen are shown. o, RBC counts. The numbers of mice per treatment are shown in each bar of each panel. All data reflect mean ± s.d. from 4 (a–h) or 3 (i–o) independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, statistical significance relative to normal mice. †P < 0.05, ††P < 0.01, statistical significance between single mutants and compound mutants. †††P < 0.001, statistical significance between SCf mutant mice and control mice after bleeding or pregnancy.

Pregnant Tcf21creER; Cxcl12fl/fl females also had significantly reduced splenic cellularity and splenic erythropoiesis relative to pregnant Cxcl12fl/fl controls, without any changes in bone marrow hematopoiesis (Extended Data Fig. 8i–x).

The splenic EMH niche after blood loss

Repeated bleeding significantly increased erythropoiesis and myelopoiesis in the red pulp, increasing spleen cellularity, HSC number, and progenitor numbers relative to non-bled controls (Extended Data Fig. 9a–i). Just as in Cy+G-CSF-treated mice, SCF-GFP was largely expressed by endothelial cells and perivascular stromal cells in the red pulp while Cxcl12-expr was expressed by a subset of SCF-GFP+ stromal cells (Extended Data Fig. 9j–l). Blood loss induced the proliferation of these cells, significantly expanding their numbers (Extended Data Fig. 9m–o). In bled mice, Tcf21-Cre/ER recombined in red pulp PDGFRα+LepR+ stromal cells, but not in bone marrow and rarely in liver (Extended Data Fig. 9p–v). Vav1-Cre recombined in 66 ± 4.2% of spleen endothelial cells, mainly in the red pulp, but only in 7.5 ± 4.0% of bone marrow endothelial cells and 25 ± 5.8% of liver endothelial cells (Extended Data Fig. 10a–h).

Bled Tcf21creER, Scfβ/β mice or Vav1-cre; Scfβ/β mice did not differ from bled Scfβ/β controls in bone marrow cellularity (Fig. 5i), or the numbers of HSCs (Fig. 5j), GMPs, CMPs or MEPs in the bone marrow (Extended Data Fig. 10i–l). In contrast, bled Tcf21creER, Scfβ/β mice and Vav1-cre; Scfβ/β mice each had significantly lower RBC counts, spleen cellularity, and numbers of HSCs, GMPs, CMPs, MEPs, myeloid and erythroid cells in the spleen as compared to bled Scfβ/β controls (Fig. 5i, j and Extended Data Fig. 10m–p). Tcf21+ stromal cells and endothelial cells are thus necessary for EMH in the spleen and for the expansion of erythropoiesis after bleeding.

Endothelial and Tcf21+ stromal cells had additive effects on splenic EMH and the recovery of RBC counts after bleeding. Bled Vav1-cre; Tcf21creER, Scfβ/β mice had similar bone marrow cellularity and numbers of HSCs in the bone marrow as bled Scfβ/β controls (Fig. 5i, j). However, they had significantly reduced RBC counts, spleen cellularity, and numbers of HSCs, MEPs and erythroid cells in the spleen as compared to bled Scfβ/β mice, bled Vav1-cre; Scfβ/β mice, and bled Tcf21creER, Scfβ/β mice (Fig. 5k–n and Extended Data Fig. 10p).

Bled Tcf21creER; Cxcl12fl/fl mice also had significantly reduced cellularity, MEPs and erythroid cells in the spleen as well as significantly reduced RBC counts as compared to bled Cxcl12fl/fl controls, without any differences in bone marrow haematopoiesis (Extended Data Fig. 10q–e).
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**Supplementary Information** is available in the online version of the paper.

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**Author Contributions** C.N.I. identified the cre alleles used in this study and analysed Scf and Cxcl12 conditional knockout mice after Cy+ G-CSF treatment. B.O.Z. characterized the stromal cells in the spleen and analysed Scf and Cxcl12 conditional knockout mice after blood loss and pregnancy. M.A. generated and characterized the α-catulinGFP mice. M.M.M. analysed HSC localization in the spleen. Z.Z. performed all statistical analyses. J.R. examined spleen histology. C.N.I., B.O.Z., M.A., M.M.M. and S.J.M. designed the experiments and interpreted the results. C.N.I., B.O.Z. and S.J.M. wrote the manuscript.

**Author Information** Microarray data have been deposited in the Gene Expression Omnibus under accession number GSE71288. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to S.J.M. (sean.morrison@utsouthwestern.edu).
MethOdS

Antibodies including anti-CD45.2 (104), anti-CD45.1 (A20), anti-Gr1 (8C5), injected to ammonium chloride/potassium red cell lysis before antibody staining. Donor mice with EMH were transplanted along with 3 × 10^6 splenocytes from retro-orbital venous sinus of anaesthetized mice. Sorted doses of splenocytes from 540 rad (total 1,080 rad) delivered at least 2 h apart. Cells were injected into the irradiated using an XRAD 320 X-ray irradiator (Precision X-Ray) with two doses of Adult recipient mice were irradiated. Sampled 15 min before being killed. Samples were analysed using a FACSAria or FACSCanto II flow cytometer or a Leica SP8 confocal microscope equipped with a resonant scanner. Three-dimensional images were achieved with Bitplane Imaris v.7.7.1 software.

Deep imaging of spleens. Spleens were harvested and fixed for 4 h in 4% PFA at 4°C. Since the spleen capsule is highly autofluorescent, spleens were sectioned perpendicular to the long axis into 300-μm-thick sections using a Leica VT1000S vibratome. These 300-μm sections were fixed for an additional 2 h in 4% PFA and blocked overnight in staining solution (10% dimethylsulfoxide (DMSO), 0.5% IgePal630 (Sigma) and 5% donkey serum (Jackson ImmunoResearch) in PBS). All staining steps were performed in staining solution on a rotator at room temperature. Spleen sections were stained for 3 days in primary antibodies, washed overnight in several changes of PBS then stained for 3 days in secondary antibodies. The tissues were incubated in BABB for 3 h to overnight with several exchanges of fresh BABB. Spleen sections were mounted in BABB between two coverslips and sealed with silicone (Premium waterproof silicone II clear; General Electric). We found it necessary to clean the BABB of peroxides (which can accumulate as a result of exposure to air and light) by adding 10 g of activated aluminium oxide (Sigma) to 40 ml of BABB and rotating for at least 1 h, then centrifuging at 2,000 g for 10 min to remove the suspended aluminium oxide particles. Images were acquired using a Zeiss LSM780 confocal microscope with a Zeiss LD LCI Plan-Apochromat 1.40×/1.10 μm working distance. Images were taken at 512 × 512 pixel resolution with 2 μm Z-steps, pinhole for the internal detector at 47.7 μm. Random spots were inserted into images by generating randomized X, Y, and Z coordinates using the random integer generator at http://www.random.org.

Splenectomy. After mouse anaesthesia by ketamine/xylazine, a ventral midline incision was made and the peritoneum was breached. The splenic blood vessels were ligated with an absorbable suture (4-0 vicryl). The splenic vessels were cut distal to the suture and the spleen was removed. The vessels were cauterized and the abdomen was sutured with non-absorbable sutures (3-0 Tevdek III). Buprenorphine was administered every 12 h for 3 days to minimize postoperative pain and mice were maintained with ampicillin-containing water to avoid infection. Complete blood counts were measured one month after the surgery.

Induction of EMH by bleeding. EMH was induced by repeated bleeding over a 2-week period according to a published protocol. Briefly, 4–6-month-old mice were bled via the tail vein five times, every 3 days, removing approximately 250 μl of blood each time, then the mice were killed for analysis 2 days after the last bleed. Western blot. Approximately 30,000 CD45+ Ter119+ VE-cadherin+splenic endothelial cells were flow cytometrically sorted into 50 μl of 66% trichloroacetic acid (TCA) in water. Extracts were incubated on ice for at least 15 min and centrifuged at 16,100 × g at 4°C for 10 min. Precipitates were washed in acetone twice and the dried pellets were solubilized in 5% urea, 2% Triton X-100, and 1% diethiothreitol (DTT). Protein concentrations were determined on a 12% Bis-Tris polyacrylamide gels (Invitrogen) and transferred to PVDF membrane (Millipore). The blots were incubated with primary antibodies overnight at 4°C and then with secondary antibodies. Blots were developed with the SuperSignal West Femtochemiluminescence kit (Thermo Scientific). Primary antibodies used: rabbit-anti-SFC (Abcam, 1:1,000) and mouse-anti-actin (Santa Cruz, clone AC-15, 1:20,000).

Quantitative real-time PCR. Cells were sorted directly into Trizol (Life Technologies). Total RNA was extracted according to the manufacturer's instructions. Total RNA was reverse transcribed using SuperScript III Reverse Transcriptase (Life Technologies). Quantitative real-time PCR was performed 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: Scf: 5'-GCGA AAAAACTAGATCCTTTACTCCTGA-3' and 5'-CATAAATGGGTGTTTGTGACATCGACTCTTG-3'; Il7r: 5'-GCTTTTTCCAGCCCTTTCTTT-3' and 5'-CTCTCGATGCTGTCGCAA-3'.
Gene expression profiling. Three independent samples of 5,000 spleen Scf-GFP+VE-cadherin− spleen stromal cells and two independent samples of 5,000 unfractionated spleen cells were flow cytometrically sorted into Trizol. Total RNA was extracted, amplified, and sense strand cDNA was generated using the Ovation Pico WTA System V2 (NuGEN) according to the manufacturer’s instructions. cDNA was fragmented and biotinylated using the Encore Biotin Module (NuGEN) according to the manufacturer’s instructions. Labelled cDNA was hybridized to Affymetrix Mouse Gene ST 1.0 chips according to the manufacturer’s instructions. Expression values for all probes were normalized and determined using the robust multi-array average (RMA) method.

Statistical methods. Panels in all figures represented multiple independent experiments performed on different days with different mice. Sample sizes were not based on power calculations. No randomization or blinding was performed. No animals were excluded from analysis. Variation is always indicated using standard deviation. For analysis of the statistical significance of differences between two groups we generally performed two-tailed Student’s t-tests. For analysis of the statistical significance of differences among more than two groups, we performed repeated measures one-way analysis of variance (ANOVA) tests with Greenhouse–Geisser correction (variances between groups were not equal) and Tukey’s multiple comparison tests with individual variances computed for each comparison. To assess the statistical significance of differences in fetal mass between paired control and mutant mice (Fig. 5j and Extended Data Fig. 8v), we performed a two-way ANOVA.

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Extended Data Figure 1 | Cy+21 d G-CSF treatment induces EMH in the spleen and deletion of Scf from LepR+ cells significantly reduces the number of HSCs in the bone marrow and the spleen after induction of EMH. a,b. Staining with anti-laminin antibody distinguished the vasculature of red pulp (RP) from white pulp (WP). The red pulp and white pulp were marked by clusters of Ter119+ cells (red) and CD3+ cells (blue), respectively. Dashed line depicts the boundary between red pulp and white pulp (representative images from 3 mice in 3 independent experiments). c. Spleen sections of the same magnification show the enlargement of the spleen after induction of EMH by Cy+21 d G-CSF. These are the same images as in Fig. 1a–d, adjusted to reflect the same magnification. d–f. Imaging of thick spleen sections from ScfGFP; Cxcl12DsRed mice after the induction of EMH by Cy+21 d G-CSF. e. High-magnification view of the boxed area in d. Dashed lines depict the boundaries between white pulp and red pulp. Arrow indicates the central arteriole in the white pulp around which stromal cells expressed Cxcl12-DsRed (representative images from 3 mice from 3 independent experiments). f, g. Haematoxylin and eosin (H&E) staining showing the increase in haematopoiesis in the spleen after induction of EMH using Cy+G-CSF (+EMH, g) as evidenced by the presence of megakaryocytes (arrows; n = 3 mice per condition from 3 independent experiments). h–n. Cy+G-CSF treatment significantly increased spleen cellularity (h), as well as the numbers of HSCs (i), MEPs (j), frequencies of colony-forming progenitors (k), numbers of Ter119+ erythroid cells (l) and Gr-1+ Mac-1+ myeloid cells (m) in the spleen but not the number of B220+ or CD3+ lymphoid cells (n). The numbers of mice per treatment are shown in each bar of each panel. Each panel shows mean ± s.d. from five independent experiments. o, p. Scf-GFP (o) and Cxcl12-DsRed (p) fluorescence by spleen stromal cells before (−EMH) and after induction of EMH (+EMH) using Cy+G-CSF. q, r. The frequencies (q) and absolute numbers (r) of Scf-GFP+ VE-cadherin+ endothelial cells and Scf-GFP+ VE-cadherin− stromal cells significantly increased upon induction of EMH by Cy+21 d G-CSF (+EMH). s. Spleens from Lepr+/+; R26GFPmice showed Tomato expression was primarily in the stromal cells of the white pulp. Although most Scf-GFP+ expression was in endothelial cells and perivascular stromal cells of the red pulp (Fig. 1a–d), some Scf-GFP+ stromal cells were in the white pulp, most of which appeared to express LepR. Dashed line depicts the boundary between red pulp and white pulp (representative images of 6 mice from 4 independent experiments). t. Flow cytometric analysis of enzymatically dissociated spleen cells from Lepr+/+; R26GFP; ScfGFP mice showed that only a small minority of non-endothelial Scf-GFP+ cells were positive for Tomato (n = 3 mice from 3 independent experiments). u. Tomato+ CD45+ Ter119+ stromal cells in the spleens of Lepr-cre; R26GFPmice expressed PDGFR-α, PDGFR-β, Sca-1 and LepR (n = 3 mice from 3 independent experiments). v. Percentage of all CFU-F colonies formed by enzymatically dissociated spleen cells from Lepr+/+; R26GFP mice that expressed Tomato. Macrophage colonies were excluded by staining with anti-CD45 antibody (n = 4 mice from 3 independent experiments). w. Lepr+/+, ScfGFP− mice had significantly fewer HSCs in the bone marrow than wild-type and ScfGFP− controls before induction of EMH (n = 4 mice per genotype per time point mice from 4 independent experiments). NT, not treated. x, y. Lepr+/+, ScfGFP− mice displayed significantly lower spleen cellularity (x) and HSC number (y) in the spleen than wild-type and ScfGFP− controls after induction of EMH with cyclophosphamide plus 4 days of G-CSF. The numbers of mice per treatment are shown in each bar. Data represent mean ± s.d. from 4 independent experiments. h–n, q, r. The statistical significance of differences was assessed using two-tailed Student’s t-tests (**P < 0.001). w–y. The statistical significance of differences between genotypes was assessed using repeated measures one-way ANOVAs with Greenhouse–Geisser correction and Tukey’s multiple comparison tests with individual variances computed for each comparison. *P < 0.05, **P < 0.01, statistical significance relative to wild-type (Scf+/+). †P < 0.05, ††P < 0.01, statistical significance between Scf−/− and Lepr+/+, Scf−/−.
Extended Data Figure 2 | Scf is expressed by most endothelial cells but not by Tcf21+ perivascular cells in the liver; Cy+21 d G-CSF does not significantly change the recombination pattern of Tcf21-Cre/ER in the spleen, bone marrow or liver. a–i, To identify Cre alleles that recombine in spleen, but not bone marrow, stromal cells we assessed the gene expression profile of spleen Scf-GFP+ VE-cadherin+ stromal cells (Extended Data Table 1). Nestin, NG2 (also known as Cspg4) and Prx1 were low or undetectable (data not shown). Nestin-Cre31, NG2-Cre32, NG2-Cre/ER33, and Prx1-Cre34 did not recombine widely or specifically in Scf-GFP+ stromal cells in the spleen (data not shown). Pdgfra and Pdgfrb were expressed by spleen Scf-GFP+ stromal cells but neither Pdgfra-Cre/ER (ref. 35) nor Pdgfrb-Cre (ref. 36) recombined efficiently (data not shown). Sm22 (also known as Tagln), Myh11, Smα (also known as Acta2) and Tcf21 were significantly more highly expressed by spleen than bone marrow Scf-GFP+ stromal cells (Extended Data Table 1). Sm22-Cre (ref. 37), Myh11-Cre (ref. 38) and Smα-Cre/ER (ref. 39) recombined in few spleen Scf-GFP+ stromal cells (data not shown). However, Tcf21-Cre/ER recombined in perivascular stromal cells in the spleen but not bone marrow (Fig. 2). a–c, Under normal conditions, Scf-GFP was expressed by most VE-cadherin+ endothelial cells (arrowheads in a) but not by Tcf21+ stromal cells (arrows in a) in the liver (n = 3 mice from 3 independent experiments). d, e, EMH induced by Cy+21 d G-CSF did not alter the general distribution (d) or perivascular localization (e) of Tomato+ cells in the spleens of Tcf21cre/ER; R26tdTomato mice as compared to normal mice (Fig. 2a, d). f, g, Tomato expression was undetectable in the bone marrow of Tcf21cre/ER; R26tdTomato mice after Cy+G-CSF treatment irrespective of whether the bone marrow was analysed by whole-mount imaging (f) or flow cytometry (g). h, i, EMH induced by Cy+G-CSF did not significantly change the frequency (h) or perivascular localization (i, arrows) of Tomato+ cells in the livers of Tcf21cre/ER; R26tdTomato mice. d–i, n = 3 mice from 3 independent experiments.
Extended Data Figure 3 | Deep imaging of HSCs in the spleen; deletion of Scf or Cxcl12 from Tcf21-expressing stromal cells in the spleen reduced peripheral blood cell counts but did not affect bone marrow haematopoiesis. a, b, The vast majority of c-Kit+ haematopoietic progenitors localized adjacent to Tcf21-expressing stromal cells in the red pulp of the normal spleen (n = 3 mice from 3 independent experiments). c, d, Three-hundred-micrometre-thick sections of spleen before (c) and after optical clearing (d). e, f, Deep imaging of α-catulin-GFP+c-Kit+ HSCs in cleared spleen segments from Tcf21creER; R26tdTomato; α-catulinGFP mice. A representative high-magnification image of an α-catulin-GFP+c-Kit+ HSC surrounded by Tomato+ stromal cells (e). f, Low-magnification view of a digitally reconstructed 300-μm-thick spleen fragment with α-catulin-GFP+c-Kit+ HSCs identified by large yellow spheres. Note that actual HSCs would be smaller than the yellow spheres but would not be visible at this magnification (n = 3 mice from 3 independent experiments). g–m, Tcf21creER; Scffl/fl and Scffl/fl control mice were treated with tamoxifen then examined 1 month later without further treatment (not treated (NT)) or after treatment with cyclophosphamide plus 4, 8, or 21 days of G-CSF to induce EMH. Data show WBC (g), RBC (h) and PLT counts (i), numbers of CMPs (j), GMPs (k) and MEPs (l) in the bone marrow and numbers of GMPs in the spleen (m). n, Platelet counts of sham-operated and splenectomized mice that were treated with Cy21d G-CSF 1 month after surgery. o–u, Tcf21creER; Cxcl12fl/fl mice and littermate controls (Cxcl12fl− or Cxcl12fl−) were treated with tamoxifen then examined 1 month later without further treatment (NT) or after treatment with cyclophosphamide plus 4, 8, or 21 days of G-CSF to induce EMH. Data show WBC (o), RBC (p) and PLT counts (q), numbers of CMPs (r), GMPs (s) and MEPs (t) in the bone marrow and numbers of GMPs in the spleen (u). The numbers of mice per treatment are shown in each panel. All data reflect mean ± s.d. from 3 independent experiments. Two-tailed Student’s t-tests were used to assess statistical significance (*P < 0.05, **P < 0.001).
Extended Data Figure 4 | Conditional deletion of Scf or Cxcl12 with Tcf21-Cre/ER, or Scf with Vav1-Cre, does not significantly affect the frequency or morphology of stromal cells in the spleen, irrespective of EMH induction. a–g. Irrespective of whether the mice were treated with Cy+21 d G-CSF, conditional deletion of Scf from Tcf21 cells did not significantly change the frequency of VE-cadherin+ endothelial cells (a) or PDGFR-β+ perivascular stromal cells (b), Scf transcript levels in endothelial cells (c), or the morphology or density of blood vessels in the spleen (d–g). h–n. Irrespective of whether the mice were treated with Cy+G-CSF, conditional deletion of Cxcl12 from Tcf21 cells did not significantly change the frequency of VE-cadherin+ endothelial cells (h) or PDGFR-β+ perivascular stromal cells (i), Scf transcript levels in perivascular stromal cells (j), or the morphology or density of blood vessels in the spleen (k–n). o–u. Irrespective of whether the mice were treated with Cy+G-CSF, conditional deletion of Scf using Vav1-Cre did not significantly change the frequency of VE-cadherin+ endothelial cells (o) or PDGFR-β+ perivascular stromal cells (p), Scf transcript levels in perivascular stromal cells (q), or the morphology or density of blood vessels in the spleen (r–u). Scf transcript levels in flow cytometrically isolated cells were normalized to β-actin and then compared to whole spleen cells (c, j and q). The data reflect mean ± s.d. from 3 mice per genotype per condition in 3 independent experiments. Two-tailed Student's t-tests were used to assess statistical significance.
Extended Data Figure 5 | Vav1-Cre recombines efficiently and specifically in spleen endothelial cells but poorly in bone marrow or liver endothelial cells. a, Tomato<sup>+</sup>CD45<sup>-</sup>Ter119<sup>-</sup> cells in Vav1-cre; tdTomato mice were uniformly positive for VE-cadherin and negative for PDGFR-β (n = 3 mice from 3 independent experiments). b, Vav1-Cre recombined in most spleen endothelial cells but in few bone marrow endothelial cells, irrespective of Cy<sup>+</sup>G-CSF treatment (+EMH). c, Scf transcript levels were significantly reduced in endothelial cells from the spleen but not from bone marrow or liver in Vav1-cre; Scf<sup>fl/−</sup> mice as compared to Scf<sup>fl/−</sup> mice. The Scf transcript level was normalized to β-actin. d, Western blot showed lower SCF protein levels in splenic endothelial cells from Vav1-cre; Scf<sup>fl/−</sup> mice as compared to Scf<sup>fl/−</sup> mice. SCF abundance was assessed relative to actin by Image J software (n = 3 mice per genotype from 3 independent experiments). e–h, In the bone marrow Vav1-Cre recombined in a minority of endothelial cells, including some sinusoidal (arrows in h) and some arteriolar (arrowheads in h) endothelial cells, that expressed little Scf-GFP by flow cytometry (f, g). The data reflect mean ± s.d. from 3 mice per genotype in 3 independent experiments. i–k, Vav1-Cre recombined inefficiently in liver endothelial cells. Most Tomato<sup>+</sup> cells in the liver of Vav1-cre; R26<sup>tdTomato</sup>; ScfGFP mice were VE-cadherin<sup>+</sup> and Scf-GFP<sup>+</sup> (i; arrows in k) but these cells accounted for only 26 ± 4.2% of Scf-GFP<sup>+</sup> cells by flow cytometry (i, j) and confocal microscopy (k, n = 3 mice from 3 independent experiments). Two-tailed Student’s t-tests were used to assess statistical significance.
Extended Data Figure 6 | EMH induced by Cy+G-CSF does not significantly change the recombination pattern of Vav1-Cre in the spleen, bone marrow, or liver but deletion of Scf from endothelial cells in spleens with EMH reduces blood cell counts without affecting bone marrow haematopoiesis. a, After EMH induced by Cy+21 d G-CSF, Vav1-Cre-recombined cells were predominantly in the red pulp (a) and co-localized with VE-cadherin cells (b) in the spleen. c–f, After EMH induced by Cy+21 d G-CSF, Vav1-Cre-recombined cells remained rare in the bone marrow (c, d) and liver (e, f; n = 3 mice from 3 independent experiments). g–s, Vav1-cre; Cxcl12<sup>fl/fl</sup> mice and Cxcl12<sup>fl/fl</sup> controls were treated with Cy+4–21 d G-CSF to induce EMH. Data show WBC (g), RBC (h), and platelet (PLT) (i) counts, numbers of CMPs (j), GMPs (k) and MEPs (l) in the bone marrow as well as numbers of HSCs (m), CMPs (n) and MEPs (o) in the spleen as well as bone marrow cellularity (p) and numbers of HSCs (q), CMPs (r) and MEPs (s) in one femur and one tibia. The data represent mean ± s.d. from 3 (Cy+4 d G-CSF treatment) and 5 (Cy+21 d G-CSF treatment) independent experiments. The number of mice per treatment is indicated on each bar. Two-tailed Student’s t-tests were used to assess statistical significance. t–z, Vav1-cre; Scf<sup>fl/fl</sup> mice and Scf<sup>fl/fl</sup>, Scf<sup>fl/fl</sup> controls were treated with Cy+4–21 d G-CSF to induce EMH. Data show WBC (t), RBC (u), and platelet (PLT) (v) counts, numbers of CMPs (w), GMPs (x) and MEPs (y) in the bone marrow as well as numbers of GMPs in the spleen (z). Note that after 21 days of G-CSF both Scf<sup>fl/fl</sup> and Vav1-cre; Scf<sup>fl/fl</sup> mice showed significantly lower CMP numbers relative to Scf<sup>fl/fl</sup> mice but their CMP numbers were not significantly different from each other (w), indicating that CMP numbers in the bone marrow were not influenced by Scf deletion from spleen endothelial cells. The data represent mean ± s.d. from 3 (no treatment (NT)), 3 (4 days), 3 (8 days), and 8 (21 days) independent experiments. The number of mice per treatment is indicated on each bar. The statistical significance of differences among genotypes was assessed using repeated measures one-way ANOVAs with Greenhouse–Geisser correction and Tukey’s multiple comparison tests with individual variances computed for each comparison. *P < 0.05, **P < 0.01, statistical significance relative to Scf<sup>fl/fl</sup> controls. †P < 0.05, ††P < 0.01, statistical significance between Scf<sup>fl/fl</sup> and Vav1-cre; Scf<sup>fl/fl</sup>.
Extended Data Figure 7 | Pregnancy induces EMH and the proliferation of endothelial cells and stromal cells in the spleen without significantly changing the recombination pattern of Tcf21-Cre/ER in the spleen, bone marrow or liver. a–v, Pregnant female mice were at gestation day 18.5. a, b, H&E staining showed increased haematopoiesis in the spleens of pregnant mice (b) as evidenced by the presence of megakaryocytes (arrows; n = 3 mice per condition from 3 independent experiments). c–i, Pregnancy significantly increased spleen cellularity (c), as well as the numbers of HSCs (d), MEPs (e, f), Ter119<sup>+</sup> erythroid cells (g) and Gr-1<sup>+</sup>Mac-1<sup>+</sup> myeloid cells (h) in the spleen but not the number of B220<sup>+</sup> or CD3<sup>+</sup> lymphoid cells (i). j, k, During pregnancy, Scf-GFP was expressed by VE-cadherin<sup>+</sup> endothelial cells and VE-cadherin<sup>−</sup>stromal cells (j) while Cxcl12-DsRed was expressed by a subset of the VE-cadherin<sup>−</sup>Scf-GFP<sup>+</sup>stromal cells (j, k). l, Whole-mount imaging of a thick spleen section from a pregnant Scf<sup>GFP</sup>; Cxcl12<sup>DsRed</sup> mouse (representative images from 3 mice in 3 independent experiments). m, n, In the spleen, the numbers of Scf-GFP<sup>+</sup> cells (m) and Cxcl12-DsRed<sup>+</sup> cells (n) significantly increased upon bleeding. o, Endothelial and stromal cells in the spleen proliferated after bleeding. BrdU was administered to Scf<sup>GFP</sup> mice or Cxcl12<sup>DsRed</sup> mice for 18 days, beginning in pregnant mice after the plug was observed. The number of mice per treatment is indicated on each bar. Each panel shows mean ± s.d. from 3 independent experiments. Two-tailed Student’s t-tests were used to assess statistical significance (***P < 0.001, **P < 0.01, *P < 0.05). p–r, Pregnancy did not alter the general distribution (p), perivascular localization (q) or surface marker expression (r; PDGFR<sup>−</sup>B<sup>−</sup> and LepR<sup>−</sup>) of Tomato<sup>+</sup> cells in the spleens of Tcf21<sup>Cre/ER</sup>; R26<sup>tdTomato</sup> mice. s, t, Tomato expression remained undetectable in the bone marrow of pregnant Tcf21<sup>Cre/ER</sup>; R26<sup>tdTomato</sup> mice. u, v, During pregnancy, Tcf21-Cre/ER recombined in rare perivascular cells in the liver. p–v, n = 3 mice per genotype from 3 independent experiments.

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Extended Data Figure 8 | Conditional deletion of Cxcl12 from Tcf21<sup>Cre</sup> stromal cells impairs EMH in the spleens of pregnant mice without significantly affecting bone marrow haematopoiesis. a–x, Four-to-six-month-old female mice that had been treated with tamoxifen at least 2 months before were mated with normal wild-type males. Normal females and pregnant females at gestation day 18.5 were analysed. a–d, Conditional deletion of Scf from Tcf21<sup>Cre</sup> cells did not significantly affect the numbers of GMPs (a), CMPs (b), MEPs (c), Ter119<sup>+</sup> (erythroid), Gr-1<sup>+</sup> Mac-1<sup>+</sup> (myeloid), CD3<sup>+</sup> (T) and B220<sup>+</sup> (B) cells (d) in one femur or one tibia. e–f, Conditional deletion of Scf from Tcf21<sup>Cre</sup> cells significantly reduced GMPs (e), Ter119<sup>+</sup> erythrocytes and Gr-1<sup>+</sup> Mac-1<sup>+</sup> myeloid cells (f) in the spleen. g, h, Conditional deletion of Scf from Tcf21<sup>Cre</sup> cells did not significantly affect WBC (g) or platelet counts (h). i–n, Conditional deletion of Cxcl12 from Tcf21<sup>Cre</sup> cells did not significantly affect bone marrow cellularity (i), or the numbers of HSCs (j), GMPs (k) CMPs (l), MEPs (m), Ter119<sup>+</sup> (erythroid), Gr-1<sup>+</sup> Mac-1<sup>+</sup> (myeloid), CD3<sup>+</sup> (T) and B220<sup>+</sup> (B) cells (n) in the bone marrow. o–w, Spleen cellularity (o) and numbers of HSCs (p), GMPs (q), CMPs (r), MEPs (s), Ter119<sup>+</sup> (erythroid), Gr-1<sup>+</sup> Mac-1<sup>+</sup> (myeloid), CD3<sup>+</sup> (T) and B220<sup>+</sup> (B) cells (t) in the spleen and WBC (u), platelet (v) and RBC counts (w) in the blood. x, Conditional deletion of Cxcl12 from Tcf21<sup>Cre</sup> cells in the spleens of pregnant mothers did not significantly affect fetal mass. The numbers of mice per treatment are shown in each bar within each panel. Each panel shows mean ± s.d. from 3 independent experiments. a–w, The statistical significance of differences among genotypes was assessed using a repeated measures one-way ANOVA with Greenhouse–Geisser correction along with Tukey’s multiple comparison tests with individual variances. *P < 0.05, **P < 0.01, ***P < 0.001, statistical significance relative to normal mice. †P < 0.05, ††P < 0.01, †††P < 0.001, statistical significance between Scf mutant mice and control mice after bleeding.
Extended Data Figure 9 | Bleeding induces EMH and the proliferation of endothelial cells and stromal cells in the spleen without significantly changing the recombination pattern of Tcf21-Cre/ER in the spleen, bone marrow, or liver. a, b, H&E staining showed an increase in haematopoiesis in the spleen after repeated bleeding (b; bled) as evidenced by the presence of megakaryocytes (arrows; n = 3 mice per condition from 3 independent experiments). c–i, Bleeding significantly increased spleen cellularity (c), as well as the numbers of HSCs (d), MEPs (e, f), and the numbers of Ter119+ erythroid cells (g) and Gr-1+Mac-1+ myeloid cells (h) in the spleen but not the number of B220+ or CD3+ lymphoid cells (i). j, k, After EMH induced by bleeding, Scf-GFP was expressed by VE-cadherin+ endothelial cells and VE-cadherin− stromal cells (j) while Cxcl12-DsRed was expressed by a subset of the VE-cadherin− Scf-GFP+ stromal cells (j, k). l, Whole-mount imaging of a thick spleen section from a ScfGFP; Cxcl12DsRed mouse after bleeding (representative images from 3 mice in 3 independent experiments). m, n, The numbers of Scf-GFP+ cells (m) and Cxcl12-DsRed+ cells (n) significantly increased upon bleeding. o, Endothelial and stromal cells in the spleen proliferated after bleeding. BrdU was administered to ScfGFP mice or Cxcl12DsRed mice for 15 days beginning after the first bleeding. The numbers of mice per treatment are shown in each bar in each panel. Each panel shows mean ± s.d. from three independent experiments. Two-tailed Student’s t-tests were used to assess statistical significance (***P < 0.001, **P < 0.01). p–r, Bleeding did not alter the general distribution (p), perivascular localization (q) or surface marker expression (r, PDGFR-β and LepR−) of Tomato+ cells in the spleens of Tcf21cre/ER; R26TdTomato mice. s, t, Tomato expression remained undetectable in bone marrow from Tcf21cre/ER; R26TdTomato mice after bleeding. u, v, After bleeding, Tcf21-Cre/ER recombined only in rare perivascular cells in the liver (p–v; n = 3 mice per genotype from 3 independent experiments).
Blood loss does not significantly change the recombination pattern of Vav1-Cre in the spleen, bone marrow, or liver; conditional deletion of Cxcl12 from Tcf21+ spleen stromal cells in bled mice impairs EMH without significantly affecting bone marrow haematopoiesis. a–e. Four-to-six-month-old mice with the indicated genetic backgrounds were repeatedly bled over a 2-week period. a–h. After EMH induced by blood loss, Vav1-Cre recombined efficiently in VE-cadherin+ endothelial cells in the red pulp of the spleen (a–c) but poorly in the bone marrow (d–i) and liver (g, h), similar to what we observed under normal conditions (see Fig. 4a–c and Extended Data Fig. 5b) (a–h; n = 3 mice from 3 independent experiments). i–n. Conditional deletion of Scf using Tcf21-Cre/ER and/or Vav1-Cre did not significantly affect bone marrow cellularity (q), or the numbers of HSCs (r), GMPs (s) CMPs (t), MEPS (u), Ter119+ (erythroid), Gr-1+ Mac-1+ (myeloid), CD3+ (T) and B220+ (B) cells (v) in one femur and one tibia from bled mice. w–b′. Conditional deletion of Cxcl12 from Tcf21+ spleen cells significantly reduced spleen cellularity (w), and the numbers of MEPS (a′) and erythrocytes (b′) in the spleens of bled mice. Conditional deletion of Cxcl12 from Tcf21+ spleen cells did not significantly affect the numbers of HSCs (x), GMPs (y), or CMPs (z) in the spleens of bled mice. c–e′. Conditional deletion of Cxcl12 from Tcf21+ spleen cells significantly reduced RBC (c′) but not WBC (d′) or platelet counts (e′) in the blood of mice that had been repeatedly bled. q–e′. Data represent mean ± s.d. from 3 independent experiments. The numbers of mice per treatment are shown in each bar in each panel. Statistical significance of differences among genotypes was assessed using a repeated measures one-way ANOVA with Greenhouse–Geisser correction along with Tukey’s multiple comparison tests with individual variances. *P < 0.05, **P < 0.01, ***P < 0.001, statistical significance relative to normal mice. †P < 0.05, ††P < 0.01, †††P < 0.001, statistical significance between Scf mutant mice and control mice after bleeding.
Extended Data Table 1 | Genes that are significantly more highly expressed by Scf-GFP+ stromal cells in spleen as compared to bone marrow

| Gene         | Gene name                              | Unigene | Spleen Scf-GFP+ | BM Scf-GFP+ | Fold change |
|--------------|----------------------------------------|---------|-----------------|-------------|-------------|
| Coch         | Coagulation factor C homolog           | Mm.21325| 12.1±0.3        | 6.6±0.0     | 45.4        |
| Ccl21a       | Chemokine (C-C motif) ligand 21A       | Mm.458815| 12.5±0.1        | 7.1±0.4     | 41.1        |
| Acta2        | Actin, alpha 2, smooth muscle, aorta   | Mm.213025| 11.9±0.3        | 6.7±0.1     | 35.2        |
| Cxcl13       | Chemokine (C-X-C motif) ligand 13      | Mm.10116| 11.8±0.3        | 6.8±0.2     | 30.3        |
| Tcf21        | Transcription factor 21                | Mm.16497| 11.3±0.6        | 6.6±0.0     | 25.9        |
| Clca1        | Chloride channel calcium activated 1   | Mm.454553| 11.1±0.3        | 6.6±0.0     | 22.5        |
| Ifl27/2a     | Interferon, alpha-inducible protein 27 like 2A | Mm.271275| 11.3±0.2        | 7.2±0.4     | 16.6        |
| Pln          | Phospholamban                          | Mm.34145| 10.7±0.1        | 6.6±0.0     | 16.3        |
| Parn1        | Prostate androgen-regulated mucin-like 1 | Mm.5002| 10.8±0.3        | 6.8±0.1     | 16          |
| Fn1          | Fibronectin 1                          | Mm.193099| 10.7±0.4        | 6.8±0.2     | 14.9        |
| Col14a1      | Collagen, type XIV, alpha 1            | Mm.297899| 10.4±0.2        | 6.7±0.1     | 12.6        |
| Nr4a1        | Nuclear receptor subfamily 4, group A, 1 | Mm.119| 10.5±0.6        | 7.0±0.3     | 11.2        |
| Agtr1a       | Angiotensin II receptor, type 1a       | Mm.35062| 10.7±0.7        | 7.3±0.6     | 11          |
| Fos          | FBJ osteosarcoma oncogene              | Mm.246513| 11.4±0.4        | 8.0±0.4     | 10.7        |
| Atp1b2       | ATPase, Na+/K+ transporter, beta 2     | Mm.235204| 10.6±0.2        | 7.2±0.2     | 10.6        |
| Tnxb         | Tenascin XB                            | Mm.290527| 9.9±0.5         | 6.6±0.0     | 9.5         |
| Myh11        | Myosin, heavy polypeptide 11, smooth muscle | Mm.250705| 10.7±0.7        | 7.5±0.2     | 9.4         |
| Hspb1        | Heat shock protein 1                   | Mm.13849| 10.8±0.7        | 7.6±0.2     | 9.3         |
| Clca2        | Chloride channel calcium activated 2   | Mm.20897| 9.8±0.4         | 6.6±0.0     | 8.8         |
| Tagln        | Transgelin                             | Mm.283283| 10.4±0.5        | 7.3±0.9     | 8.6         |
| Nr2f2        | Nuclear receptor subfamily 2, group F, 2 | Mm.158143| 10.7±0.3        | 7.6±0.3     | 8.5         |
| Mstn1        | Musculoskeletal, embryonic nuclear protein 1 | Mm.220895| 10.8±0.5        | 7.7±0.7     | 8.2         |
| Aspn         | Asporin                                | Mm.383216| 9.7±0.6         | 6.6±0.0     | 8.2         |
| Sparcl1      | SPARC-like 1                           | Mm.29027 | 12.1±0.1        | 9.1±0.4     | 8.1         |

Significance was considered as >8 fold and P < 0.015. Data show mean ± s.d. for log2 transformed expression values (n = 3 independent samples per cell population). Maximal background expression was considered to be 6.6 (log2(100)); all expression values below this threshold were set to 6.6 for purposes of calculating fold change. Two-tailed Student’s t-tests were used to assess statistical significance. Data for bone marrow Scf-GFP+ stromal cells are from ref. 19.