SUPPLEMENTAL INFORMATION

Rhythmic modulation of the hematopoietic niche through neutrophil clearance

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SUPPLEMENTAL DATA

Figure S1. Phenotypic characterization of aged neutrophils, related to Figure 1.

(A) The CD62L^{LO} phenotype in PEdKO and *Fut7*$^{-/-}$ mice is due to impaired extravasation of aging neutrophils. The phenotype of WT-DsRed neutrophils was determined in WT, PEdKO and *Fut7*$^{-/-}$ partner mice one month after establishing parabiosis. Histograms are representative of 7 parabiotic pairs.

(B) Scheme for BrdU labeling of endogenous neutrophils. A single injection of 5-BrdU (2.5 mg/mouse) labels cycling precursors in the bone marrow (right scheme), and allows tracking of mature neutrophils (Eash et al., 2009) which can be detected in the circulation 48h after treatment (Basu et al., 2002). Antibodies to P- and E-selectin extend the time that labeled neutrophils can be tracked *in vivo* by preventing their extravasation.

(C) Injection of 1 mg Sulfo-NHS-LC Biotin labels circulating Ly6G^{+} neutrophils and allows identification of freshly released bone marrow cells after 1 hour as CD62L^{HI} (grey gate), whereas CD62L^{LO} neutrophils are Biotin^{+} (red gate), indicating that they were already present in blood at the time of labeling. The dot plot shows CD62L levels and biotinylation in Ly6G^{+} blood neutrophils. Data is representative of 5 mice.

(D) Percentages of Annexin-V binding to CD62L^{LO} and CD62L^{HI} Ly6G+ neutrophils present in the blood of PEdKO mice were determined by flow cytometry and analyzed as indicated for panel F below. n=4 mice per group. Bars represent mean ± s.e.m.

(E) CD62L^{LO} neutrophils are not apoptotic. Levels of Mcl-1 and cleaved caspase 3 (cC3) as determined by western blotting of lysates from sorted CD62L^{LO} and CD62L^{HI} neutrophils from blood, or total neutrophils from BM treated or not with Roscovitine. Representative blot from 2 experiments.

(F) Differential expression of surface markers on CD62L^{HI} and CD62L^{LO} neutrophils in blood. PEdKO mice were treated with 100 mg/kg fucoidan to induce mobilization of “fresh” neutrophils from the BM (Frenette and Weiss, 2000). 30 minutes later blood

samples were obtained and analyzed by flow cytometry for the expression of CD62L together with the indicated surface receptors. For analysis of relative levels of expression, mean fluorescence intensity values were calculated from the cells with the 20% highest levels of CD62L ($CD62L^{HI}$; grey dots) or with the 50% lowest levels of CD62L ($CD62L^{LO}$; red dots) within each sample. Bars represent mean ± s.e.m. Statistical comparisons were made using paired t-test analysis. *P<0.05, **P<0.01, ***P<0.001.

(G) Relative values of forward-scatter (FSC) and side-scatter (SSC) properties of the $CD62L^{HI}$ and $CD62L^{LO}$ subsets as determined by flow cytometry and illustrated in the dot plot. Note that y-axis values start at 100 and report small but significant differences. n=6-8 mice per marker. Bars represent mean ± s.e.m. Statistical comparisons were made using paired t-test analysis. *P<0.05, ***P<0.001.

Figure S2. Modulation of hematopoietic niches and HPC trafficking by neutrophil depletion, and requirement of receptors involved in neutrophil trafficking, related to Figure 2.

(A) Flow cytometry analyses of BM from *Cxcl12-Gfp* and *Nestin-Gfp* mice used to quantitate the number of GFP^{LO} cells, CAR cells and $Nestin^{+}$ niche cells. Bars show an increase in the absolute number and frequency of GFP^{LO} cells, but not $Nestin^{+}$ cells, in neutrophil-depleted (1A8) mice. Data from n= 4-5 mice per group. Bars represent mean ± s.e.m. *P<0.05; n.s., not significant.

(B) Effect of neutrophil depletion on the percentage of BrdU+ GFP+ cells in the BM of *Cxcl12-Gfp* reporter mice. Bars represent mean ± s.e.m. *P<0.05.

(C) Effect of neutrophil depletion on the expression of *Cxcl12* in GFP+ cells present in the BM of *Cxcl12-Gfp* reporter mice. Bars represent mean ± s.e.m. **P<0.01; n.s., not significant.

(D) Experimental scheme and effect of neutrophil depletion in the number of primitive HSC in blood. Populations enriched in stem cells ($Lineage^{NEG}$ $Sca\text{-}1^{+}$ $cKit^{+}$, or LSK cells) and $Lineage^{NEG}$ $Sca\text{-}1^{+}$ $cKit^{+}$ $Thy1.2^{+}$ $Flk2^{NEG}$ (LSKTF) were identified by flow cytometry and their numbers per ml of blood calculated from the total blood counts. n= 8 mice per group. Bars represent mean ± s.e.m. *P<0.05.

(E) Effect of neutrophil depletion on *Fut7*$^{-/-}$ mice. Levels of progenitors in the blood of untreated wild-type mice are shown for comparison. Data from n=8 mice. Bars represent mean ± s.e.m. **P*<0.05; n.s., not significant.

(F) Differential requirement of functional selectin ligands and CXCR4 for neutrophil migration to bone marrow, spleen and liver. Neutrophils from *Fut7*$^{-/-}$ or MR4 donors lack functional selectin ligands or CXCR4, respectively (see also Figure S3D below). The migration of experimental neutrophils (*Fut7*$^{-/-}$, MR4 or WT control; blue) was assessed relative to DsRed^{+} WT competitors (red) co-injected into the same recipient mouse. n=6 mice per group. Bars represent mean ± s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.001.

Figure S3. Modulation of hematopoietic niches and HPC trafficking by neutrophil transfer and characterization of MR4 mice, related to Figure 3.

(A) Experimental scheme and effect of neutrophil transfer in the number of primitive HSC (identified as in panel S2D) in blood. Note that the baseline levels of LSKTF differ with the neutrophil-depletion (panel S2D) experiments because blood samples were collected at different times of the day: ZT5 and ZT1, respectively. n= 9 mice per group. Bars represent mean ± s.e.m. **P*<0.05.

(B) Only progenitor cells (CFU-C) are significantly altered by transfer of neutrophils, but not total leukocytes (WBC), neutrophils (PMN) or lymphocytes (Lymp). Mice were injected at ZT13 and bled at ZT1. n=6-9 mice per group. Bars represent mean ± s.e.m. **P*<0.05.

(C) Numbers of blood CFU-C present in the WT or mutant partners of WT-GFP mice after one month in parabiosis. Values correspond to the partners of mice shown in Figure 3D. Dotted lines show baseline CFU-C levels in those mutants before parabiosis. Data are from 5 to 12 parabionts per group. Bars represent mean ± s.e.m. *, *P*<0.05, ****P*<0.001.

(D) Blood neutrophil counts and plasma levels of G-CSF in wild-type, PEdKO and *Fut7*-/- mice. Age- and sex-matched mice from all groups were bled at ZT5 for analysis. n = 8-15 mice per group. Bars represent mean ± s.e.m. ****P*<0.001; n.s., not significant.

(E) Mice with myeloid-specific deficiency in CXCR4 were generated by crossing *LysM*-Cre and *Cxcr4*$^{flox/flox}$ mice (MR4). Expression of CXCR4 is specifically lost in myeloid (Ly6G^{+} neutrophils), but not non-myeloid (CD11b^{NEG}) leukocytes. Light grey histograms are samples from Cre^{NEG} control mice; red histograms are samples from MR4 mice; dashed histograms show staining with an isotype control.

(F) Number of total and CD62L^{LO} neutrophils in the blood of control (MR4 Cre^{NEG}) and MR4 (MR4 Cre^{+}) mice. Data from 5 (control) to 13 (MR4) mice. Bars represent mean ± s.e.m. ***P<0.001.

(G) Representative micrograph and frequency of hypersegmented neutrophils in the blood of MR4 and control mice. Data from 5 mice per group. Scale bar, 10 μm. Bars represent mean ± s.e.m. ***P<0.001.

(H) Flow cytometric analysis of the expression of CD62L and CD11b on Ly6G^{+} neutrophils in the blood of control and MR4 mice. Plots are representative of 5 mice per group.

Figure S4. Analysis of neutrophil homing into BM and engulfment by BM macrophages, related to Figure 5.

(A) *Cxcl12-Gfp* mice prepared for imaging as indicated in the Procedures section were injected with 50 μg Alexa 647-conjugated Dextran (10 KDa) and 10 min later imaged by multiphoton microscopy. Second harmonic generation allowed visualization of the bone (blue), CAR cells were visualized by GFP expression (green), and dextran-labeled cells were visualized in the far-red channel (white). Cell-like structures were visualized as the fluorescent dextran diffused into the parenchyma (left, untreated mouse). These elements completely disappeared in mice treated with clodronate 24h before imaging (right), indicating that the dextran-labeled cells were macrophages. In clodronate-treated mice (right), dextran delineates the vasculature. Bar, 50 μm.

(B) Flow cytometry plots used for analysis of the homing and engulfment of CD62L^{HI} and CD62L^{LO} neutrophils. Equal numbers of CFSE-labeled CD62L^{HI} or CD62L^{LO} neutrophils were injected into wild-type recipient mice and their presence in the BM analyzed 4 hours later. BM cells were stained for Gr1 and F4/80 to discriminate between homed (Gr1^{HI} F4/80$^{LO/NEG}$; top panels) and engulfed (Gr1^{NEG} F4/80+; bottom

panels) cells. Control panels show staining in uninjected control mice. Plots are representative from 5 mice per group.

(C) Physiological homing and engulfment of neutrophils in the BM. BM cells from WT mice that were in parabiosis with *LysM-Gfp* partners were analyzed to quantify Gr-1^{HI} F4/80$^{LO/NEG}$ GFP+ homed neutrophils (top panels), and Gr-1^{NEG} F4/80+ GFP+ engulfing cells (bottom panels). Numbers in the plots indicate the mean percentage of GFP+ neutrophils or engulfing macrophages. Data from 3 parabiotic pairs.

(D) Specific depletion of BM macrophages by clodronate. Representative cytometry plots of BM cells obtained 11 days after treatment with saline (white bars) or clodronate liposomes (colored bars). The different regions identify neutrophils (green), monocytes (purple) and macrophages (red regions in lower panels). Data from 9 mice per group. Bars represent mean ± s.e.m. ***P<0.001; n.s., not significant.

Figure S5. Circadian and neutrophil-induced changes in gene expression of *Cxcl12*, LXR and LXR-target genes in the BM, related to Figure 6.

(A) Relative expression of the genes encoding for LXRα and β, in various populations of BM cells, including total BM (tBM), stromal cells (CD45^{NEG} TER119^{NEG} CD31^{NEG}), endothelial cells (CD45^{NEG} TER119^{NEG} CD31+), LSK progenitors, neutrophils (PMN), CXCL12-producing cells (CD45^{NEG} TER119^{NEG} CD31^{NEG} GFP^{HI} and GFP^{LO}) and macrophages (Macs). Data is from 2 pools for each cell type. Bars represent mean ± s.e.m.

(B) Analyses of the transcript levels of *Cxcl12* and *Abca1* in the BM of WT mice at different times of the day. The expression of both genes follows circadian fluctuations that are shifted by 8h. Shaded areas represent periods of darkness. n=5-9 mice per time point. Dots represent mean ± s.e.m.

(C) Niche-modulating macrophages are present in LXRdKO mice. Number of CFU-C in the blood of WT and LXRdKO mice treated or not with clodronate. n=4-7 mice per group. Bars represent mean ± s.e.m. **P<0.01, ***, P<0.001.

(D) Neutrophil transfer mobilizes progenitors in *Fut7*$^{-/-}$ mice. Levels of CFU-C in the blood of untreated wild-type mice are shown for comparison. n=8 mice. Bars represent mean ± s.e.m. *P<0.05, **P<0.01.

(E) Analysis of the relative expression of *Abca1* in different BM cell populations: total BM (tBM), hematopoietic cells (LSK and PMN), CXCL12-producing cells and macrophages. Data from 4 pooled samples. Bars represent mean ± s.e.m. ***P<0.001.
(F) Expression of *Abca1* and *Mertk* in BM macrophages sorted from control (rIgG) or neutrophil-depleted mice (1A8). n=3 mice per group. Bars represent mean ± s.e.m. **P<0.01.
(G) Strategy for purification of engulfing and non-engulfing $Gr1^{NEG}$ F4/80+ macrophages from the BM of WT mice in parabiosis with WT-DsRed partners.
(H) Expression of *Abca1* and *Mertk* in BM macrophages from parabiotic mice that have engulfed or not blood-borne cells. n = 3 pairs per group. Bars represent mean ± s.e.m. ***P<0.001.
(I) Representative micrographs of $Gr\text{-}1^{NEG}$ F4/80+ macrophages sorted on the basis of gain of DsRed signal. Fluorescent signal and Wright-Giemsa staining are shown. Scale bars, 10 μm.

Movie S1. Homing to the bone marrow occurs in areas enriched in CAR cells, related to Figure 4. DsRed+ BM cells (red) were injected into *Cxcl12-Gfp* mice and imaged by combined multiphoton and confocal microscopy through a 30 μm-thick section. Four hours after injection, all cells that homed into the BM (red) were found associated with areas enriched in CAR cells (green). Vessels and macrophages (white) were visualized by injection of fluorescently-conjugated dextran immediately before imaging.

Movie S2. *In vivo* imaging of aged neutrophils, niche cells and macrophages within the bone marrow, related to Figure 5. DsRed+ BM-derived aged neutrophils (white) were injected into *Cxcl12-Gfp* mice and imaged in time-lapse for the following 150 min through a 30 μm-thick section. Intravascular neutrophils can be seen moving rapidly. Extravasated neutrophils move short distances and establish frequent interactions with marrow macrophages (red), which are identified by the uptake of fluorescent dextran injected at the beginning of the experiment (see Figure S4A). The

enlarged area allows visualization of a single neutrophil interacting with several macrophages, but not with CAR cells (green). Bone appears in light blue.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Extended Experimental Procedures

Mice. All experiments were performed on 6-12 week old C57BL/6 male and female mice. Chow and water were available *ad libitum*. Mice deficient in *Fut7,* in both *Sele* and *Selp* (PEdKO), and in LXRα and β (*Nhr1h3*$^{-/-}$*Nhr1h2*$^{-/-}$) have been previously described (Frenette et al., 1996; Maly et al., 1996; Peet et al., 1998). The following knock-in mice were also used: *Cxcr4*$^{f/f}$ (Nie et al., 2004); *LysM*-Cre (Clausen et al., 1999), *LysM-Gfp* (Faust et al., 2000) and *Cxcl12-Gfp* (Sugiyama et al., 2006) mice. Mice with myeloid deficiency in CXCR4 (MR4 mice) were generated by crossing *LysM*-Cre and *Cxcr4*$^{f/f}$ mice. Transgenic mice expressing GFP or DsRed under the control of the *β-actin* promoter or the *Nestin* promoter (*Nestin-Gfp*) were also used. All mice were in the C57BL/6 background. Mice were maintained on a 12h light/12h darkness lighting schedule. Mice were housed in a specific pathogen-free facility at Centro Nacional de Investigaciones Cardiovasculares. Experimental procedures were approved by the Animal Care and Ethics Comittee of the Centro Nacional de Investigaciones Cardiovasculares.

Parabiosis. To generate parabiotic pairs, we followed previously described procedures (Wright et al., 2001). Anesthetized mice were shaved at the corresponding lateral aspects and matching skin incisions were made from the olecranon to the knee joint of each mouse, and the subcutaneous fascia was bluntly dissected to create about 0.5 cm of free skin. The olecranon and knee joints were attached by a single 5-0 polypropylene suture and tie, and the dorsal and ventral skins were approximated by continuous suture. A single dose of flunixin meglumine (Schering-Plough, Segré, France) was injected subcutaneously in each partner at the end of the surgical procedure (1 mg/kg). One month after surgery blood samples were obtained from each of the partners for analysis of hematopoietic progenitors.

Progenitor assays in culture. Blood was collected by retro-orbital bleeding, and 100 μl of blood placed over a Lympholyte bed (Cedarlane Labs; Burlington, USA) and

centrifuged to obtain mononuclear cells. Cells were added to semisolid media containing 1.25% methylcellulose (Sigma-Aldrich, Saint Louis USA), 30% fetal bovine serum (StemCell Technologies, Vancouver, Canada), 1% deionized bovine serum albumin (BSA), 10^{-4}M 2-mercaptoethanol, and conditioned medium (12.7% v/v) from the WEHI3 cell line (containing IL-3), HM-5 cell line (containing GM-CSF) and BHK/MKL cell line stably transfected to produce the secreted form of murine Kit Ligand/SCF. Cultures were plated in duplicates in 35 mm culture dishes (NUNC A/S; Roskilde, Denmark) and incubated at 37°C in 5% CO_2. The numbers of CFU-Cs were scored on day 6 or 7 using an inverted microscope.

Long-term competitive reconstitution assays. To compare the long-term reconstitution capacity of HSC present in the blood of neutrophil-depleted and control mice, we mixed equal volumes of blood from CD45.1+ B6.SJL mice (3 control-treated donors) and C57BL/6 CD45.2+ mice (3 neutrophil-depleted donors) that were age- and sex-matched. Four hundred microliters of this mixture were then supplemented with 10^6 BM cells from a DsRed-Tg donor to allow survival of the recipients, and intravenously injected into lethally irradiated (13 Gy in two split doses) DsRed-Tg recipient mice. Recipient mice were bled monthly for cytometric analysis until week 16. The relative reconstitution activity present in each blood donor was measured by comparing the number of $DsRed^{NEG}$ CD45.1+ and $DsRed^{NEG}$ CD45.2+ leukocytes in the recipients' blood.

Purification of bone marrow and blood neutrophils. BM cells from femurs and tibiae were harvested from C57BL/6 mice by flushing with HBSS containing 2 mM EDTA. PMN were purified using Percoll 62% (GE Healthcare) and erythrocytes lysed using a hypotonic buffer. A purity of 90±1% neutrophils was achieved using this protocol. To induce senescence, neutrophils from BM were cultured 6 to 20 h in complete media (RPMI supplemented with 10% fetal bovine serum plus penicillin/streptomycin) (Furze and Rankin, 2008). Following culture, the purity of Ly6G-positive neutrophils and CXCR4 and CD62L levels were determined by flow cytometry.

To prepare neutrophils from blood, PEdKO or WT mice treated with anti-P/E-selectins antibodies (clones RB40 and 9A9, respectively; 25 μg/mouse at -2 and -1 days) were bled. Blood was incubated in 2% methylcellulose for 40 min at room temperature. Leukocyte-containing supernatants were then pooled and lysed in hypotonic buffer. Total blood leukocytes were labeled with antibodies against CD3, CD4, B220, CD71, CD115 and Siglec-F (see Table below); unlabeled cells were purified by cell sorting, resulting in ≥95% pure PMN. Sorting was performed in a MoFlo Legacy (Beckman Coulter) or FACSAria II (BD Biosciences; San Jose, CA). Alternatively, total blood leukocytes were incubated with biotinylated antibodies against Ter119, B220, CD3, CD115, CD117, CD4, CD8, CD49b and F4/80 followed by incubation with streptavidin-conjugated paramagnetic beads (BD Biosciences). Non-bound cells were recovered after exposure to a magnet and analyzed by flow cytometry. Purities were 90±2% for WT and 95±1% for PEdKO donors.

***In vivo* labeling of neutrophils**. Blood leukocytes were labelled by a single intravenous injection of 1 mg Sulfo-NHS-Biotin (Sigma, Thermo Scientific; Rockford, USA) in PBS into WT mice. Mice were bled 1h later and labeled with antibodies against Ly6G and CD62L, followed by incubation with Alexa 450-conjugated streptavidin. For metabolic labeling with 5-Bromodeoxyuridine (BrdU), mice treated with anti-P/E-selectin antibodies were injected intraperitoneally with a single dose of 2.5 mg (BD Biosciences). Blood samples were collected from mice at the indicated times and stained for Ly6G and CD62L, followed by fixation and intracellular labeling of BrdU using an APC-conjugated anti-BrdU antibody as per the manufacturer's instructions (BD Biosciences).

Flow cytometry. For macrophage staining, BM cells were stained for Gr1, CD115, F4/80, and analyzed by flow cytometry as indicated. For quantification of HSPC, BM cells were stained for lineage markers (CD3, B220, TER119, Mac-1 and Gr-1), Sca-1 and c-Kit (LSK population). Short-term and long-term stem cells were further discriminated within the LSK fraction by staining for Thy1.2 and Flk2. In all cases, DAPI (Sigma) was used to identify dead cells, which were excluded from analysis. For

detection of blood HSPC, 250 μl of blood was loaded onto a Lympholite gradient and mononuclear fractions were stained as described above.

For estimation of CXCL12-expressing niche cells, femoral plugs were collected at the indicated times, and incubated with 1U/ml liberase (Roche Applied Science, Mannheim, Germany) and 12 mU/ml DNAse I (Sigma) in HBSS for 30 min at 37ºC. Total BM cells were then stained with biotinylated antibodies against TER-119, CD31 and CD45 followed by incubation with DyLight 649-conjugated streptavidin (Jackson Immunoresearch; Suffolk, UK). High or intermediate levels of GFP in the CD45/TER119/CD31-negative fraction were used to discriminate CAR cells from the GFP^{LO} fraction.

In all procedures, cells were incubated with the indicated antibodies for 15 min at 6ºC. Cytometric analyses were performed using a FACS Canto flow cytometer equipped with DIVA software (BD Biosciences). Data were analyzed with the DIVA or FlowJo (TreeStar Inc.; Ashland, OR) softwares. All experiments were conducted at the CNIC-Cellomics Unit. The fluorescently-labeled or biotinylated antibodies used are listed below.

List of antibodies used for flow cytometry

Antibody	Source	Clone	Antibody	Source	Clone
CD3ε-biotin	BD	145-2C11	Thy1.2-APC	eBioscience	53-2.1
CD11b-biotin	BD	M1/70	FcγR-PE	eBioscience	93
CD11b- FITC	BD	M1/70	Flk2-PE	eBioscience	A2F10
B220-biotin	BD	RA3-6B2	CXCR2-PE	R&D	242216
Gr1-biotin	BD	RB6-8C5	CD47-FITC	BD	MIAP301
CD45-biotin	eBioscience	30-F11	CD4-biotin	eBioscience	GK1.5
Ter119-biot	BD	Ter119	CD8a-biotin	eBioscience	53-6.7
CD115-PE	eBioscience	AFS98	CD115-biotin	eBioscience	AFS98

CD31-APC	eBioscience	390	F4/80-biotin	eBioscience	BM8
CD62L-APC	BD	MEL-14	CD117-biotin	eBioscience	2B8
CD62L-PE	BD	MEL-14	CD117-biotin	eBioscience	2B8
CXCR4-PE	eBioscience	2B11	CD49b-biotin	eBioscience	DX5
F4/80-APC	AbD Serotec eBiosciences	A3.1 BM8	CD49d-biotin	In-house	PS/2
Siglec-F-PE	BD	E50-2440	CD31-Biotin	eBioscience	390
Gr1-FITC	eBioscience	RB6-8C5			
Sca1-FITC	eBioscience	D7	SA-PE	eBioscience	
c-kit PE-Cy7	eBioscience	2B8	SA-eF450	eBioscience	
AnnexinV-PE	BD		SA-APCeF780	eBioscience	

Abreviations: SA, Streptavidin

Proliferation of CXCL12-GFP^{+} BM cells. Control or neutrophil-depleted *Cxcl12-Gfp* mice were injected i.p with 2.5 mg/mouse of BrdU. Bones and bone marrow plugs were digested as previously described and single cell suspensions were analyzed by flow cytometry as indicated above. Proliferative cells were detected as CD45/TER119/CD31-negative GFP^{LO} or GFP^{HI} BrdU^{+} cells.

Preparation and immunostaining of BM femoral sections. 3.5x10^{6} neutrophils obtained from the BM of DsRed mice were senesced in culture and injected intravenously into *Cxcl12-Gfp* mice. 6 to 8 hours later mice were perfused with 2% paraformaldehyde (PFA) and tibiae and femurs were removed and fixed in a 2% PFA solution overnight at 4ºC, rehydrated in 30% sucrose for 48 h, and snap frozen in OCT (Tissue Tek; Torrance, CA). Whole longitudinal single cell-thick (5 μm) femoral cryosections were obtained using a Leica Cryostat and the Cryojane tape transfer system (Leica Microsystems). Sections were blocked in PBS containing 10% donkey

serum and the biotin/avidin blocking kit (Vectashield; Burlingame, CA) and stained with rat anti-CD169 (Abcam; Cambridge, MA), followed by biotinylated anti-rat IgG and DyLight 649-conjugated streptavidin (Jackson Immunoresearch).

Laser scanning cytometry analysis. Laser Scanning Cytometry was performed using an iCys Research Imaging Cytometer system (CompuCyte; Westwood, MA), equipped with 4 lasers (405, 488, 561 and 633 nm) and 4 detectors with the following filter sets: 450/40, 521/15, 575/50 and 650/LP. Sections were scanned with a 10x objective using the 405 nm laser to generate low resolution images of the DAPI stained nuclei and obtain a general view of the BM. Regions of interest were subsequently scanned with a 40x dry objective lens at a spatial resolution of 0.25 mm stage step size to create high resolution field images of the whole BM cavity. Lasers were selected to detect CXCL12-GFP$^+$ stromal cells, DsRed$^+$ neutrophils and DyLight 649-labeled CD169$^+$ cells. Detector levels were set according to background fluorescence of unlabeled or isotype control stained sections, which were always scanned in parallel to stained samples. Distances between cells or bone structures were measured manually for every homed neutrophil using the profile tool of the iCys software.

***In vivo* cell depletion.** For depletion of blood neutrophils, 50 μg of anti-Ly6G antibody (1A8 clone; BioXCell; West Lebanon, NH) was injected intraperitoneally for 2 consecutive days resulting in >90% reduction in blood neutrophil counts (637 ± 41 vs 55 ± 12 cells/μl for control vs. 1A8-treated, respectively; $P<0.001$); the levels of lymphocytes and monocytes in blood, or macrophages in BM were not affected by this treatment (not shown). The number of neutrophils in BM was also unaffected by the anti-Ly6G treatment. A similar schedule was followed with an anti-CD4 antibody (clone GK1.5, BD Biosciences) for depletion of CD4-positive lymphocytes (65% reduction; 2108 ± 297 vs 717 ± 85 cells/μl for control vs. GK1.5-treated, respectively; $P<0.01$) or with rIgG (Sigma) as control. For macrophage depletion, 100-150 μl of clodronate-loaded liposomes were intravenously injected per mouse 10 days prior to experiments. The efficiency of macrophage depletion in BM was assessed by flow cytometry at day 11 (Figure S4D).

Homing assays.

Wild-type vs. Fut7- or CXCR4-deficient neutrophils. Blood leukocytes from *Fut7*-/-, MR4 and DsRed-Tg donors were prepared as described above. Cells from experimental and WT donors were mixed in equal proportions and intravenously injected into GFP-transgenic or *LysM-Gfp* recipient mice. Neutrophils were allowed to home for 3 hours before collecting blood and, after perfusion with PBS containing 2 mM EDTA, liver, spleen and BM were collected. Cell suspensions prepared from these organs were processed and stained for DyLight 649-conjugated Ly6G (1A8), and analyzed by flow cytometry. The initial mixture of cells was used to control for the input ratios between the two donor cell populations.

Homing of LSK cells to the BM. Wild-type mice were depleted of circulating neutrophils as indicated above, and 10^7 BM mononuclear cells obtained from DsRed-transgenic mice were injected intravenously. Cells were allowed to home for 2 hours and the femoral BM cells of the recipient were collected and stained for LSK as described above. Donor-derived LSK cells were identified within the LSK fraction by expression of DsRed by flow cytometry. The absolute number of homed donor LSK cells was determined after correcting by the total number of cells in the recipients' femurs.

Homing and engulfment of CD62L^{LO} and CD62L^{HI} neutrophils. CD62L^{LO} and CD62L^{HI} neutrophils were purified from the blood of PEdKO mice or wild-type mice treated with AMD3100, respectively, as described. Cells were stained with 10 μM 5(6)-Carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE; Invitrogen) at a concentration of 7.5 x 10^6 cells/ml PBS containing 0.1% BSA. Recipient wild-type mice were injected intravenously with equal numbers of CFSE-labeled CD62L^{LO} or CD62L^{HI} cells, and after 4 hours the mice were perfused. BM cell suspensions were processed and stained for F4/80 and Gr-1, and analyzed by flow cytometry. Homed neutrophils were defined as Gr-1^{HI}/F4/80$^{LO/NEG}$ CFSE+ cells. Macrophages were gated as Gr-1^{NEG}/F4/80+ and defined as having engulfed a homed neutrophil if they were positive for CFSE. Uninjected control mice were used to set the appropriate gates.

Epifluorescence intravital microscopy of calvarial BM. We performed multichannel fluorescence intravital imaging (MFIM) of the calvarial BM of anesthetized mice as previously described (Hidalgo et al., 2002). All images were acquired with an Axio Examiner microscope (Zeiss, Germany) equipped with a Coolsnap HQ2 camera and analyzed with Slide Book software (Intelligent Imaging Innovations; Denver, CO). $5x10^6$ 32D cells were labeled with CFSE and injected into recipient mice treated with saline or 50 μg of 1A8 antibody for 2 days. Cells were allowed to circulate for 4h before imaging. The number of 32D cells adhered to the BM sinusoids in captured fields (0.601 mm^2) was assessed immediately after injection of 10μg of rhodamine 6G (Sigma) to allow visualization of the BM microvasculature.

Intravital multiphoton microscopy of calvarial BM. *Cxcl12-Gfp* mice were anesthetized and immobilized on a custom-made holder, and positioned for imaging in a Zeiss LSM780 microscope (Zeiss). 30 min before imaging mice were intravenously injected with 2-5 million $DsRed^+$ neutrophils (BM- or blood-derived). 50 μg of Alexa 647-conjugated dextran (10 KDa; Invitrogen) was also injected to allow initial visualization of the vasculature and at later times to label macrophages, which take up the dye over the first 30 min (see Figure S4A). The presence of bone cavities enriched in both GFP^{HI} (CAR cells) and $dextran^+$ cells, which were preferred sites of homing, were selected for imaging. In another set of experiments, 32D cells were labeled with Vibrant DiD (Invitrogen) and injected into mice together with TRITC-conjugated dextran (70 KDa; Sigma) to visualize the vasculature. Second harmonic signals from bone-derived collagen fibers (Lo Celso et al., 2011) were illuminated by a Ti:Sapphire laser system (Spectra-Physics Mai Tai Deep Sea; Irvine, CA). Two-photon microscopy at 810 nm excitation was used to visualize GFP (490-534 nm detection), Alexa 647/DiD (633 nm detection) and TRITC (575-625 nm detection). $DsRed^+$ neutrophils were imaged by simultaneous confocal microscopy (561 nm excitation; 570 nm emission). Two-photon microscopy was performed at the CNIC-Microscopy Dynamic Imaging unit.

Chemokine and cytokine quantification. CXCL12 was measured from total bone marrow extracellular fluid content obtained after flushing BM from femurs in 1 ml of

PBS, using commercially available ELISA reagents (R&D Systems). Samples were diluted 1:5 in PBS. For quantification of G-CSF levels in plasma we followed a similar ELISA procedure (R&D Systems; Minneapolis; MN). Samples were diluted 1:4 in PBS.

Western blotting. Bone marrow neutrophils were purified as indicated. Apoptosis of BM neutrophils was induced by incubating cells with 20 μM R-(R)-Roscovitine (A. G. Scientific) for 4 hours in RPMI containing 10% FBS. $CD62L^{LO}$ and $CD62L^{HI}$ Ly6G+ populations of neutrophils were sorted from the blood of PEdKO mice treated with AMD3100 as described. Purified cells were lysed in RIPA buffer containing 50 mM Tris-HCl, pH8; 150 mM NaCl; 1% Triton X-100; 0.5% sodium deoxycholate; 0.1% SDS; 1 mM PMSF (Sigma) and a protease inhibitor cocktail (Sigma). Proteins from 3 x 10^5 lysed cells were separated by 12% SDS-PAGE and transferred onto PVDF membrane. Membranes were incubated overnight with antibodies for Mcl-1 (clone S-19, Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 1:100 dilution, or cleaved-caspase-3 (Asp175) (clone 5A1E; Cell Signaling Technology, Danvers, MA) at 1:1000 dilution, and then probed with horse radish peroxidase conjugated anti-rabbit antibody (1:300; GE Healthcare Life Sciences). Blots were visualized using the chemiluminescent Luminata Forte Western HRP Substrate (Millipore). For Erk1/2, membranes were stripped and reprobed with p44/42 MAPK (Erk1/2) (clone 137F5; Cell Signaling Technology) at 1:1000 dilution.

Purification of BM cell subsets. GFP^{HI} and GFP^{LO} populations were isolated from bones (femurs and tibiae) and bone marrow plugs of *Cxcl12-Gfp* mice digested in liberase/DNAse and stained with the markers indicated above before purification in a cell sorter. To isolate both stromal and endothelial cells, single cell suspensions obtained from bones and bone marrow plugs from WT mice were stained with anti-CD45, anti-Ter119 and anti-CD31 antibodies. $CD45^{NEG}Ter119^{NEG}CD31^{NEG}$ stromal cells and $CD45^{NEG}Ter119^{NEG}$CD31+ endothelial cells were sorted. Bone marrow macrophages were sorted as $Gr\text{-}1^{NEG}$F4/80+. For LSK cell sorting, $Lineage^{NEG}$ cells were enriched using biotinylated antibodies and streptavidin paramagnetic beads, and then stained as indicated in “Flow Cytometry”. Macrophages from parabiotic pairs were identified as $Gr1^{NEG}$ F4/80+, and engulfing and non-engulfing macrophages were

detected based on the presence or absence of DsRed signal, respectively, when compared to cells from a non-parabiotic control (Figs.S5G). Sorting was performed in a MoFlo Legacy or FACSAria II.

Immunofluorescence of engulfing macrophages. DsRed+ and DsRed^{NEG} macrophages were sorted from parabiotic mice, fixed in 2% paraformaldehyde, and mounted with Vectashield Mounting Medium containing DAPI (Vector Laboratories, Burlingame, CA). Image acquisition was performed with a Zeiss Axiovert 200M microscope and AxioCam using Axiovision software (Zeiss, Germany) and a 40x oil-immersion objective. In some cases, sorted macrophages were cytospun with a Cytospin 4 Cytocentrifuge (Thermo Scientific) and stained with Wright-Giemsa (Sigma).

RNA isolation, reverse transcription and real-time PCR. Total RNA was prepared with RNA Extraction RNeasy Plus Mini- or Micro-kit (Qiagen). RNA was reverse-transcribed with High-Capacity cDNA Reverse Transcription reagents kit (Applied Biosystems; Carlsbad, CA) according to the manufacturer's protocol. Real-time quantitative PCR (SYBR-green, Applied Biosystems) assays were performed with an Applied Biosystems 7900HT Fast Real-Time PCR System sequencer detector. Expression was normalized to *Hprt* and *36b4* expression. Primer sequences are listed below.

List of primers used in this study.

Primers	**Sequences 5´→3´**	**Product (bp)**	**Annealing (ºC)**
Cxcl12_Fw	CGCCAAGGTCGTCGCCG	123	62
_Rv	TTGGCTCTGGCGATGTGGC		62
Mertk_Fw	GAGGACTGCTTGGATGAACTGTA	73	63
_Rv	AGGTGGGTCGATCCAAGG		58
Abca1_Fw	GGTTTGGAGATGGTTATACAATAGTTGT	94	64

_Rv	CCCGGAAACGCAAGTCC		57
Hprt_Fw	CCTAAGATGAGCGCAAGTTGAA	86	60
_Rv	CCACAGGACTAGAACACCTGCTAA		65
36b4_Fw	ACTGGTCTAGGACCCGAGAAG	123	63
_Rv	TCCCACCTTGTCTCCAGTCT		60
C1qb_Fw	CAGGGATAAAGGGGGAGAAA	115	58
_Rv	GGACCCTTAGGGCCAACTT		59
LXRα_Fw	CAACAGTGTAACAGGCGCT	60	57
_Rv	TGCAATGGGCCAAGGC		54
LXRβ_Fw	CCCCACAAGTTCTCTGGACACT	70	64
_Rv	TGACGTGGCGGAGGTACTG		62
Col 1α1_Fw	TGTGTGCGATGACGTGCAAT	94	60
_Rv	GGGTCCCTCGACTCCTACA		60
Osteocalcin_Fw	ACCCTGGCTGCGCTCTGTCTCT	120	59
_Rv	GATGCGTTTGTAGGCGGTCTTCA		59
VE-Cadherin_Fw	GTTCAAGTTTGCCCTGAAGAA	66	59
_Rv	GTGATGTTGGCGGTGTTGT		59
Csf3_Fw	TCATTCTCTCCACTTCCG	103	54
_Rv	CTTGGTATTTACCCATCTCC		56

Abreviations: Fw, forward primer; Rv, reverse primer.

Statistics. Data are represented as mean ± standard error of the mean. All parameters tested followed normal distributions, as determined using the D'Agostino-Pearson normality test. ANOVA tests with Dunnett's multiple comparison test (for >2 groups) and

paired or unpaired 2-tailed *t*-test (for 2 groups) were used. *P* values below 0.05 were considered significant.

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