Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSC

Short title: Macrophages maintain hematopoietic stem cell niche

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ABSTRACT

In the bone marrow (BM), hematopoietic stem cells (HSC) reside in specific niches near osteoblast-lineage cells at the endosteum. To investigate regulation of these endosteal niches, we studied mobilization of HSC into the bloodstream in response to granulocyte colony-stimulating factor (G-CSF). We report that G-CSF mobilization rapidly depletes endosteal osteoblasts leading to suppressed endosteal bone formation and decreased expression of factors required for HSC retention and self-renewal. Importantly, G-CSF administration also depleted a population of trophic endosteal macrophages (osteomacs) that support osteoblast function. Osteomac loss, osteoblast suppression and HSC mobilization occurred concomitantly, suggesting that osteomac loss could disrupt endosteal niches. Indeed in vivo depletion of macrophages, in either macrophage Fas-induced apoptosis (Mafia) transgenic mice or by administration of clodronate-loaded liposomes to wild-type mice, recapitulated the i) loss of endosteal osteoblasts, ii) marked reduction of HSC-trophic cytokines at the endosteum, with iii) HSC mobilization into the blood as observed during G-CSF administration. Together these results establish that BM macrophages are pivotal to maintain the endosteal HSC niche and that the loss of such macrophages leads to the egress of HSC into the blood.

182 words
INTRODUCTION

Hematopoietic stem cells (HSC) reside in bone marrow (BM) and produce all cells required to replenish the blood and immune systems; this process is tightly regulated to maintain a steady number of leukocytes, platelets and red cells in the blood. Individual HSC undergo one of several fates: quiescence, apoptosis, cycling either to self-renew, or to generate a more committed hematopoietic progenitor cell (HPC), or migration. These fates are largely controlled by specific local micro-environments (‘niches’) in which HSC and HPC reside. While HSC niches were conceptualized in the 1970s, it is only in recent years, with the advent of mouse genetic models and live microscopy, that precise locations and nature of HSC niches have been proposed. Recent microscopy studies have shown that quiescent long-term reconstituting HSC preferentially reside within 20µm of the interface between compact bone and BM (endosteum) and 10 µm (or 2 cell diameters) from osteoblasts, in poorly perfused regions of the BM. Similarly, Lin⁻CD41⁻Sca1⁺KIT⁺CD48⁻CD150⁺ HSC isolated from the femoral endosteal regions have higher long-term reconstitution potential than phenotypically identical HSC isolated from the central BM. Functional studies support a role for osteoblast-lineage cells to maintain HSC in the BM in vivo. Transgenic mice with increased osteoblast function have more HSC in the BM whereas targeted ablation of osteoblasts in vivo results in progressive loss of HSC from the BM. Finally, targeted deletion in osteoprogenitors of Dicer-1, necessary for the maturation and processing of all microRNA, results in pancytopenia, exaggerated HSC proliferation, ineffective hematopoiesis and myelodysplasia. Thus it appears that the endosteal region and osteoblast-lineage cells provide a unique environment necessary for HSC survival, quiescence, self-renewal, and contribution to long-term hematopoiesis.

Despite significant breakthroughs in the identification of cellular and molecular components of BM HSC niches, little is known of how these niches are regulated in vivo. To gain insights in the regulation of endosteal niches, we studied therapeutic mobilization of HSC. During mobilization, HSPC (HSC and HPC) are forced to migrate and circulate in the blood, allowing the harvest of large numbers of HSC from patients for transplantation. Granulocyte colony-stimulating factor (G-CSF) is the most common mobilizing agent used in the clinic. While inhibition of endosteal osteoblasts has been reported during G-CSF treatment, the mechanisms leading to this inhibition remain unclear. While a role of sympathetic nerves innervating the BM and the bone was proposed, recent experiments using transplantation chimeras of wild-type and G-CSF receptor (GCSFR)-deficient mice
show that BM leukocytes are also required for osteoblast suppression and HSPC mobilization by G-CSF\textsuperscript{17}.

We confirm that G-CSF rapidly and transiently ablates endosteal osteoblasts\textsuperscript{17} and extend these observations by demonstrating a cessation of endosteal bone formation. Strikingly, this depletion of endosteal osteoblasts and bone formation was accompanied by a loss of BM macrophages, particularly osteoblast-supportive endosteal macrophages (osteomacs). Furthermore, depletion of BM macrophages, in particular a novel CD11b\textsuperscript{+}F4/80\textsuperscript{+}Ly6-G\textsuperscript{+} phagocytic macrophage population, in two mechanistically unrelated \textit{in vivo} models, was sufficient to suppress endosteal osteoblasts, inhibit expression of HSC-supportive cytokines at the endosteum, and elicit HSPC mobilization into the peripheral blood. This study reveals a critical role for BM macrophages in maintaining the endosteal HSC niche.

**METHODS**

Procedures were approved by the University of Queensland’s Animal Experimentation Ethics Committee.

**G-CSF Mobilization**

Dipeptidylpeptidase-1 (DPP-1) and GCSFR knock-out mice backcrossed into C57BL/6 were kindly provided by Dr C Pham and DC Link (Washington University, St Louis). All experiments were performed on 12-14 week-old skeletally mature C57BL/6 mice except G-CSF-treated control mice for protease concentration assays (Figure 7A) which were 129Sv. To assess effects of G-CSF mobilization, mice were injected twice daily subcutaneously with 125µg/kg/injection recombinant human G-CSF (a kind gift of Amgen, Thousand Oaks, CA) or saline for up to 6 consecutive days. When required, osteoclast activity was blocked by a 6 day treatment with 20µg/kg/day subcutaneous injections of zoledronate (Zometa, Novartis Pharmaceuticals Australia, North Ryde, Australia) prior to sacrifice and G-CSF was administered twice subcutaneously at 125µg/kg for the last 3 days prior to sacrifice.

To assess bone formation during mobilization, G-CSF or saline was injected from day 0 to day 5 as described above. Calcein (Sigma Chemicals, St Louis, MO) was injected intraperitoneally at a dose of 20mg/kg on days 1 and 7. Xylenol orange (Sigma Chemicals)
was injected intraperitoneally at a dose of 200mg/kg on days 4 and 10. Mice were sacrificed on day 12.

**Macrophage depletion *in vivo***

In a first model, myeloid cells were depleted from skeletally mature Macrophage-Fas-Induced Apoptosis (Mafia) transgenic mice by daily intravenous retro-orbital injection of 10 mg/kg/day AP20817 ligand (generous gift from ARIAD Pharmaceuticals Inc, Cambridge, MA www.ariad.com/regulationkits) dissolved in 4% ethanol, 10% PEG-400, 1.7% Tween-20\(^1\). Controls were Mafia mice injected with vehicle alone and wild-type mice injected with AP20817 ligand.

In a second model, clodronate (dichloromethylene bisphosphonate), a gift from Roche Diagnostics (Mannheim, Germany), was packaged into liposomes as previously described\(^1\). Empty liposomes were prepared in the same conditions in PBS without clodronate. Phagocytic macrophages were depleted *in vivo* by retro-orbitally injecting 250µL/25g clodronate-loaded liposome suspension every second day. Control mice were injected with an equivalent volume of saline or PBS-loaded liposomes.

**Tissue harvesting and RNA extraction**

At specified time-points, mice were anesthetized and 1mL blood collected into heparinized tubes by cardiac exsanguinations. Red cells were lysed from blood as previously described\(^2\). Spleens were harvested, weighed and dissociated in PBS with 2% FCS. Cells and RNA from enriched central BM, and endosteal RNA were isolated from femurs as previously described\(^3\). Tibias were fixed in PBS containing 4% paraformaldehyde overnight at 4°C and stored in 70% ethanol until embedding in methacrylate\(^4\) for undecalcified bone histomorphometry, or decalcified in 14% EDTA and embedded in paraffin for immunohistochemistry and enzyme staining.

**Quantitative real-time RT-PCR (RT-qPCR)**

RNA was extracted with Trizol, precipitated, DNase treated and reverse transcribed using random hexamers. RT-qPCR with SYBR green for osteocalcein, osterix, KL, Ang-1, VE-cadherin and fibronectin or using Taqman probes (labeled 5’ with 6-carboxyfluorescein (FAM) and 3’ with blackhole quencher-1 (BHQ-1)) for Runx2 and PTHR1 were performed following manufacturer’s instructions (ABI systems, Foster City, CA). Oligonucleotide sequences are shown in Table 1. Primers were designed to cross intron-exon boundaries to not
amplify genomic DNA, with the exception of osterix that is encoded by a single exon. A PCR from each sample prior to reverse transcription confirmed absence of genomic DNA. RNA levels were standardized by parallel RT-qPCR using primers to the house-keeping gene β2-microglobulin.

Histomorphometry
Tibial samples were embedded in methacrylate, and undecalcified 5μm sections were stained with toluidine blue and histomorphometry carried out in the trabecular bone of the proximal tibial secondary spongiosa using the Osteomeasure system (OsteoMetrics Inc, Decatur, GA)22. For mineral appositional rates, sections remained unstained and fluorochromes were visualized by epifluorescence microscopy22.

Calcein and xylenol orange mineralization fronts were visualized on a Zeiss LSM 510 Meta confocal laser scanning microscope. Calcein fluorescence was excited at 488nm with an Ar laser and emission detected through a 505-530nm filter whereas xylenol orange fluorescence was excited at 543nm with a He-Ne laser and emission detected through a 560nm filter. Each fluorophore was excited, scanned and acquired separately.

Cell counts and colony assays
Leukocytes were counted on a Sysmex KX-21 automated cell counter. For myeloid colony assays, cells were deposited in 35mm Petri dishes and covered with 1mL IMDM supplemented with 1.6% methylcellulose (high viscosity Methocell MC, Fluka), 35% FCS. Optimal concentrations of mouse IL-3, IL-6 and soluble KL were added as conditioned media from stably transfected BHK cell lines. Colonies were counted after 10 days of culture.

Competitive repopulation assays
The content of mobilized blood samples in competitive repopulating HSC was determined in competitive repopulation assays. The day prior to blood harvest, recipient congenic B6.SJL CD45.1+ mice were lethally irradiated with 11.0Gy in two split doses 3 hours apart. 50μL blood samples from 4 mobilized CD45.2+ C57BL/6 mice were pooled within each treatment group and 50μL blood aliquots were taken from this pool and mixed with 200,000 competitive whole BM cells from untreated B6.SJL CD45.1+ in a total volume of 200μL, and injected retro-orbitally into each lethally irradiated recipient. Recipients were maintained with antibiotics for the first 3 weeks post-transplant and tail bled 16 weeks post-transplant to
determine CD45.2 (test donor blood) versus CD45.1 (competitive whole BM cells) in myeloid, B and T lineages by flow cytometry using CD45.1-PE, CD45.2-FITC, B220-allophycocyanin (APC)-cyanin (CY) 7, CD11b-PECY7, CD3-biotin and streptavidin (SAV)-Pacific Blue.

**Flow cytometry**

Following flushing with PBS, enriched central BM cells were pelleted at 370xg for 5 minutes at 4°C and resuspended in CD16/CD32 hybridoma 2.4G2 supernatant to block IgGFc receptors except for myeloid progenitor stains.

HSC were stained with the biotinylated lineage antibody cocktail (CD3, CD5, B220, CD11b, Gr-1, Ter119) and biotinylated CD41 together with SAV-PB, Sca-1-PECY7, KIT-APC, CD48-FITC and CD150-PE.

Mature myeloid cells were stained with CD11b-PECY7, anti-LY-6G-PE (clone 1A8) and anti-F4/80-AlexaFluor647.

Osteoblast-lineage cells, MSC and endothelial cells were isolated from the endosteum following collagenase digestion of cleaned bone fragments as previously described. Hematopoietic cells were removed by magnetic depletion using biotinylated lineage antibodies (CD3, CD5, B220, CD11b, Gr-1, Ter119) followed by depletion with MACS beads conjugated to SAV (Miltenyi Biotech). Cells were then stained at 4°C with CD45-APCCy7, CD31-APC, CD51-PE, Sca-1-PECY7, SAV-FITC for wild-type mice or SAV-PB for col2.3CreR26R-YFP+/+ mice and sorted on the gating strategy: CD45LinCD31bright endothelial cells; CD45Lin CD31Sca-1brightCD51+ MSC, and CD45LinCD31Sca-1CD51+ osteoblast lineage cells.

All antibodies were from BD Pharmingen except CD150-PE (clone TC15-12F12.2) and anti-F4/80-AlexaFluor647 (clone CI:A3-1) from Biolegend. Data were acquired on an LSRII (BD Biosciences) or CyAn (Dako Cytomation) flow cytometers and analyzed following compensation with single color controls using FloJo software (Tree Star, Ashland, OR).

**Immunohistochemistry and TRAP enzyme histology**

Fixed and decalcified hind limb bones were embedded in paraffin and 5μm sections cut and placed on SuperFrost Plus® slides (Menzel, Germany). Sections were deparaffinized and rehydrated with xylene and graded ethanol and washed in TBS (immunohistochemistry) or deionized water (histology). F4/80 (rat anti-mouse F4/80, AbD Serotec) and osteocalcin...
(rabbit anti-mouse, Alexis Biochemicals, San Diego, USA) immunohistochemical staining was carried out as previously described\textsuperscript{26}. Specificity of staining was confirmed by comparison to serial sections stained with matched isotype control antibodies (Rat IgG\textsubscript{2b}, AbD Serotec and normal rabbit IgG, Santa Cruz, respectively). TRAP staining was performed as previously described\textsuperscript{27}. All sections were examined using an Olympus BX-51 microscope with a DP-70 digital camera and DP controller imaging software (Olympus).

**Protease concentration**

Quantification of active neutrophil elastase (NE) and cathepsin (G) BM fluids using specific chromogenic substrates, and gelatin zymopgraphy to measure matrix metalloproteinase (MMP)-9 was carried out as previously described\textsuperscript{28,29}.

**Statistics**

Significance levels were calculated using the Student’s t-test for colony assays, flow cytometry analyses, and RT-qPCR, or two-way ANOVA followed by Fisher’s PLSD post-hoc test for histomorphometric measurements.

**RESULTS**

**HSPC mobilization by G-CSF inhibits endosteal niches for HSC**

Administration of G-CSF to skeletally-mature mice increased the number of hematopoietic colony forming cells (CFC), and long-term reconstituting Lin\textsuperscript{−}CD41\textsuperscript{−}Sca1\textsuperscript{+}KIT\textsuperscript{+}CD48\textsuperscript{−}CD150\textsuperscript{+} HSC\textsuperscript{30} in the blood from day 2 of G-CSF administration (Figure 1A), reaching a plateau between days 4-6, and rapidly returned to baseline when G-CSF administration was ceased (Figure 1A). Endosteal markers of bone formation including the proportion of bone surface lined with osteoblasts (ObS/BS) or newly formed bone matrix (osteoid, OS/BS), all decreased 16- to 18-fold during mobilization but rebounded four days after cessation of G-CSF administration to two-fold above baseline (Figure 1B) then continued to decrease until day 21. Both ObS/BS and OS/BS dropped 40-50% on day 1, and 75% on day 2 of G-CSF administration whereas the number of circulating CFC, Lin\textsuperscript{−}Sca1\textsuperscript{+}KIT\textsuperscript{+} and long-term reconstituting Lin\textsuperscript{−}CD41\textsuperscript{−}Sca1\textsuperscript{+}KIT\textsuperscript{+}CD48\textsuperscript{−}CD150\textsuperscript{+} HSC significantly increased in blood only on day 2.
Messenger RNA for osteoblast-lineage-specific genes including osteocalcin, Runx2/Cbfa1 and osterix\textsuperscript{31,32} were also reduced during G-CSF treatment (Supplementary Figure 1). Osteocalcin mRNA decreased 50-fold returning to basal levels 4 days following cessation of G-CSF, paralleling the changes in ObS/BS and OS/BS. In addition, mRNA levels for PTHR1, the receptor for both parathyroid hormone (PTH) and PTH-related protein, which is expressed by osteoblasts, were also decreased 24-fold by G-CSF treatment.

To confirm a functional alteration in bone formation, labeling with the fluorescent calcium chelators calcein and xylenol orange was carried out during G-CSF treatment and recovery. These labels rapidly incorporate into bone being mineralized at the time of injection (Figure 1C). Continuous bone formation results in labeling of the bone surface with two lines of calcein (green) alternating with two lines of xylenol orange. Both mineralization and bone formation were strongly reduced on both endocortical (diaphysis) and trabecular surfaces (metaphysis) in G-CSF-mobilized mice compared to controls. In contrast, the outer cortical surface (periosteum), was labeled with well-separated multiple fluorochromes in both G-CSF-treated mice and controls, indicating that the disruption in bone formation induced by G-CSF treatment was restricted to the endosteum (i.e. surfaces in contact with BM). Quantification of calcein labels (Figure 1D) revealed that the percentage of bone surfaces on which bone formation occurred (double calcein-labeled bone surface dLS/BS) was significantly reduced by G-CSF treatment from 29±1% to 6±2%. On those few surfaces on which bone formation occurred during mobilization, the mineral apposition rate (MAR), was reduced by 30%. Together, the endosteal trabecular bone formation rate per unit bone surface (BFR/BS) was reduced by 90% in G-CSF-mobilized mice. In contrast, periosteal mineralization surface (PsMAR) was not affected by G-CSF administration. This confirms that only mineralization by osteoblasts in direct contact with the BM is inhibited during HSPC mobilization.

HSC are supported by cytokines and chemokines produced within specific niches at the endosteum, including KL, Ang-1 and CXCL12. In order to determine whether these cytokines were expressed by osteoblasts, we sorted endosteal cells into: i) CD45\textsuperscript{−} Lin\textsuperscript{−} CD31\textsuperscript{bright} endothelial cells, ii) CD45\textsuperscript{−} Lin\textsuperscript{−} CD31\textsuperscript{−} Sca-1\textsuperscript{bright} CD51\textsuperscript{+} multipotent stromal cells (MSC) with osteogenic, chondrogenic and adipogenic potential\textsuperscript{24,25}, and iii) CD45\textsuperscript{−} Lin\textsuperscript{−} CD31\textsuperscript{−} Sca-1\textsuperscript{−} CD51\textsuperscript{+} osteoblast-lineage cells\textsuperscript{6,23,24}. RT-qPCR for osteocalcin and the endothelial cell marker VE-cadherin validated this sorting strategy (Supplementary Figure 2). This strategy was further validated by analyzing these 3 populations from col2.3Cre\textsuperscript{+} R26R-YFP\textsuperscript{+/+} mice in which a Cre-inducible yellow fluorescent protein (YFP) reporter is activated only in maturing and mature osteoblasts\textsuperscript{33}. In these mice, 38±13% of cells (n=3 mice) in the osteoblast gate
were YFP⁺, while only 5±1% of cells in MSC gate were YFP⁺, and YFP⁺ cells were undetectable in the endothelial gate (Supplementary Figure 2).

We then evaluated expression of CXCL12, KL and Ang-1 in endothelial, MSC, and osteoblast-lineage cells by RT-qPCR (Figure 1E). CXCL12, a chemokine necessary for HSPC retention in the BM and HSC survival and proliferation was expressed at high levels by osteoblast-lineage cells and at much lower levels by MSC and endothelial cells. Ang-1, a cytokine essential to HSC development was also transcribed by both osteoblast-lineage cells and MSC. Finally KL, a cytokine that acts on all hematopoietic progenitors and HSC, was transcribed by both osteoblast-lineage cells and endothelial cells. These mRNAs were all expressed at much higher levels in endosteal cells compared to the whole BM population (Figure 1E). Importantly during G-CSF administration, mRNA levels of CXCL12, KL and Ang-1 were all significantly decreased at the endosteum (Figure 1F). In contrast, expression of fibronectin, an extracellular matrix protein produced by BM stromal cells, remained unaltered. Taken together these observations demonstrate that the expression of HSC-trophic cytokines are decreased within the endosteum during mobilization suggesting impaired niche function.

**HSPC mobilization is not mediated by osteoclasts**

To determine whether osteoclasts were involved in the HSC mobilization and osteoblast inhibition, histochemical staining for tartrate-resistant acid phosphatase (TRAP) and histomorphometry of trabecular bone both showed that the number of osteoclasts on endosteal surfaces (NOc/BPm) was not significantly altered during G-CSF administration (Figure 2A-B). mRNA for cathepsin K, a marker of active osteoclasts, increased 10-fold, but only 4 days after cessation of G-CSF administration (Figure 2C), indicating that any increase in osteoclast number or activity occurs only after HSPC mobilization ceased. The loss of mature osteoblasts during G-CSF treatment and egress of HSPC to blood is therefore unlikely to be dependent on osteoclast depletion; in fact, the normal coupling between osteoclast number and osteoblast number appears to be disrupted during G-CSF administration.

To confirm whether osteoclasts are necessary for G-CSF-induced mobilization of HSPC, osteoclasts were inhibited with zoledronate prior to and during G-CSF mobilization. As expected, zoledronate treatment dramatically decreased endosteal osteoclast numbers and did not prevent the reduction in osteoblast numbers in G-CSF-mobilized mice (Figure 2D-E). Mice treated with zoledronate and G-CSF showed enhanced mobilization of CFC into blood and spleen compared to mice mobilized with G-CSF alone (Figure 2F-G).
which was accompanied by a greater reduction in ObS/BS (Figure 2E). Zoledronate alone did not induce mobilization (not shown). To determine whether repopulating HSPC were also mobilized, we performed competitive repopulation assays with the mobilized blood samples. Blood from individual C57BL/6 CD45.2+ mice were pooled in equal volume within each treatment group, and 50µL of pooled blood was mixed with 200,000 competing BM cells from untreated congeneric CD45.1+ mice and transplanted into lethally irradiated CD45.1+ hosts. After transplant, zoledronate significantly increased multi-lineage chimerism from mobilized CD45.2+ blood HSC (Figure 2H). Taken together, our data confirm that osteoclasts are not required for HSPC mobilization or the osteoblast suppression associated with mobilization.

G-CSF mobilization disrupts endosteal osteomac and BM macrophage distribution

F4/80+ osteomacs reside in osteal tissues and are intercalated and/or associated with other bone lining cells26. These osteomacs form a canopy over mature osteoblasts at sites of bone formation (Figure 3B), support osteoblast function in vitro and are required for maintenance of osteoblasts in vivo26. Since macrophages39, but not osteoblasts16,23 express GCSFR, osteomacs were investigated as a candidate mediator of G-CSF effects on osteoblasts. We found F4/80+ osteomacs were reduced on endosteal surfaces from day 2 of G-CSF administration (Figure 3C). This reduction was accompanied by a clustering of F4/80+ cells in the central BM, particularly around endothelial sinuses. Between days 4 and 6 of mobilization, F4/80+ cells at the endosteum were virtually undetectable (Figure 3D).

F4/80+ cells within the endosteal region were detected again 2 days after cessation of G-CSF administration (Figure 3E). The osteomac canopy over cuboidal osteoblasts on endosteal surfaces was fully restored by day 10 (Figure 3F). Thus, the kinetics of osteomac loss parallels the loss of endosteal osteoblasts in the same animals (Figure 1).

In vivo depletion of myeloid cells in Mafia transgenic mice deregulates endosteal niches and mobilizes HSPC

To determine the contribution of BM and endosteal macrophages to the regulation of endosteal niches, we ablated c-fms expressing cells, which include most mature myeloid cells such as macrophages and granulocytes, in Mafia transgenic mice. In these mice, a c-fms promoter induces expression of a transgene containing green fluorescent protein (GFP) and a suicide fusion protein made of the FK506 binding protein and the cytoplasmic domain of Fas. Systemic administration of AP20187, dimerizes the suicide protein to cause Fas-mediated
apoptosis in c-fms-expressing cells\textsuperscript{18}. Cells expressing the transgene GFP were assessed in Mafia mouse BM by flow cytometry (Table 2). As expected, more than 85\% of BM F4/80\(^+\)Ly-6G\(^-\)CD11b\(^+\) macrophages and 90\% Ly-6G\(^+\)F4/80\(^+\)CD11b\(^+\) granulocytes were GFP\(^+\) indicating expression of the Mafia suicide transgene in both cell populations. Importantly, the Mafia transgene was not expressed in osteoblast-lineage cells, MSC or BM endothelial cells.

Administration of AP20187 ligand ablated transgene-expressing GFP\(^+\)CD11b\(^+\) myeloid cells, Ly-6G\(^+\) granulocytes and F4/80\(^+\) macrophages in the BM (Figure 4A,B,F) as previously reported\textsuperscript{18}. In addition, AP20187 treatment elicited robust CFC mobilization into the blood and spleen (Figure 4C,D). RT-qPCR analyses on endosteal RNA from treated Mafia mice revealed a 35-fold decrease in osteocalcin, 8-fold decrease in Ang-1 and 3-fold decrease in KL mRNA (Figure 4E), demonstrating impairment of endosteal HSC niches following ligand treatment. Immunohistochemistry showed depletion of BM macrophages and osteomacs on metaphyseal and diaphyseal surfaces in AP20187-treated Mafia mice (Figure 4F) and almost complete loss of osteocalcin-positive osteoblasts from trabecular and endocortical bone surfaces (Figure 4F). Osteoclasts, assessed by TRAP staining, were moderately decreased at day 5 (data not shown). None of these effects were observed in wild-type mice treated with AP20187 ligand (Figure 4A-F) demonstrating that this drug is not toxic for myeloid cells, HSPC or mature osteoblasts in the absence of the Mafia transgene. These results suggest that macrophage depletion alone is sufficient to alter endosteal HSC niches and lead to HSC mobilization into blood.

\textit{In vivo} depletion of phagocytes with clodronate-loaded liposomes disrupts endosteal niches and mobilizes HSPC

The mafia mouse model is not ideal as G-CSF concentration increases in blood during depletion and other myeloid cells are depleted in addition to macrophages. Our data were confirmed in a second mechanistically unrelated model of macrophage depletion \textit{in vivo}. Mice were injected intravenously with clodronate-loaded liposomes (clo-lip), control PBS-loaded liposomes (PBS-lip), or saline for 1-4 days. Clo-lip treatment (but not PBS-lip) specifically kills phagocytes, the only cells capable of engulfing liposomes\textsuperscript{19,40}. Although BM contains multiple monocyte/macrophage/granulocyte populations, only some were depleted following Clo-lip administration. Specifically, a macrophage population with the unusual F4/80\(^+\)Ly-6G\(^-\)CD11b\(^+\) phenotype was reduced by 95\% within 24 hours of clo-lip administration and remained depleted throughout clo-lip treatment (Figure 5A). Interestingly, BM granulocytes (Ly-6G\(^+\)F4/80\(^+\)CD11b\(^+\)) were not affected by clo-lip administration until later time-points (4
days), when their numbers eventually increased 2.5-fold above baseline. As a control, BM T cell numbers remained unaltered during clo-lip administration (not shown).

Depletion of phagocytic macrophages in the BM by clo-lip administration elicited significant mobilization of CFC and phenotypic Lin-Sca1+KIT+ HSPC within 24h (Figure 5B). While no long-term competitive repopulating HSC capable of multi-lineage engraftment were detected in the blood of control PBS-lip injected mice, blood transplantation revealed the presence of circulating long-term competitive repopulating HSC after 2 days of clo-lip administration (Figure 5C) with multi-lineage engraftment in 5 of 6 recipients (Fisher exact test p=0.008), and after 4 days of clo-lip, multi-lineage engraftment was observed in 7 of 7 recipients (Fisher exact test p=0.0006). Blood chimerism from mice mobilized with clo-lip for 4 days was 90% in recipients compared to 29±13% donor chimerism when donor mice were mobilized with G-CSF for 3 days (Figure 2H).

Administration of clo-lip also dramatically down-regulated osteocalcin, CXCL12, KL and Ang-1 mRNA in the BM over 10-fold within 24 hours, reflecting rapid disruption of niche function when BM phagocytic macrophages were ablated (Figure 6A). Immunohistochemistry showed severe depletion of endosteal osteomacs in the secondary spongiosa of the metaphysis within 24 hours of clo-lip administration (Figure 6B) and flattened morphology of osteocalcin-positive osteoblasts followed by osteoblast depletion at day 2 of clo-lip administration (Figure 6B). This time-course of depletion in osteoblast number, and bone formation after clo-lip administration was confirmed by histomorphometry (Figure 6C). TRAP staining and histomorphometry also confirmed a decrease in osteoclast number and function within 2 days of clo-lip administration (Supplementary Figure 3).

**Loss of osteoblasts with clo-lip treatment is not mediated by mature granulocytes or their proteases**

Granulocytes can release proteases such as neutrophil elastase (NE), cathepsin G (catG) and matrix metalloproteinase (MMP)-9, which cleave and inactivate the interactions maintaining HSPC within the BM, thereby initiating mobilization. As 4 days of clo-lip treatment lead to an increase in Ly-6G+F4/80CD11b+ granulocytic-lineage cells in the BM (Figure 5A), we assessed neutrophil proteases in BM fluids during clo-lip treatment. No increase in activity or concentration of these proteases was observed in BM fluids from clo-lip treated mice (Figure 7A-B) when osteoblast depletion (Figure 6B-C), decreased transcription of HSC trophic cytokines/chemokines/adhesive factors (Figure 6A), and mobilization of CFC, Lin−
Sca1+KIt+ HSPC and LT-CRC had commenced (Figure 5). At day 4 of clo-lip treatment, catG and MMP-9 concentrations were slightly increased (Figure 6A-B) in synchrony with the late expansion of Ly-6G+ granulocytic cells in the BM (Figure 5A), and increased HSPC mobilization (Figure 5B-C). The absence of significant BM protease activity during the first 2 days of clo-lip administration suggests that release of NE, catG and MMP-9 in the BM is not the primary factor inhibiting osteoblast function in response to clo-lip.

In addition, a reduction in osteoid surface and osteoblast number in response to G-CSF in DPP1-deficient mice, which mobilize normally in response to G-CSF despite complete absence of active NE and catG43, confirmed that NE and catG are not necessary for osteoblast inhibition in response to G-CSF (Figure 7C).

Finally, using GCSFR KO mice, which have very few functional granulocytes and almost no neutrophil protease activity (Figure 7E), clo-lip administration still elicited HSPC mobilization (Figure 7D) concomitant with osteoblast and osteomac depletion (Figure 7F and G). Altogether, these data show that CFC mobilization and loss of osteoblasts in response to macrophage depletion or G-CSF treatment is not initiated by proteases released from mature granulocytes in the BM.

**DISCUSSION**

Cells of the osteoblast-lineage are essential for maintenance of HSC in the BM8-12 producing cytokines, chemokines and cell-adhesion molecules that regulate HSC fate, including CXCL12, KL, Ang-1 and osteopontin. G-CSF administration transiently suppressed these cells leading to pronounced decreases in bone formation and mineralization at the endosteum, and reduced expression of CXCL12, KL and Ang-1 in the BM. This inhibition of endosteal niches coincided with mobilization of HSC and was not mediated by osteoclasts, but by a loss of osteoblast-supportive ostemacs and/or other phagocytic macrophages with an unusual F4/80+Ly-6G+CD11b+ phenotype. Indeed, in vivo depletion of macrophages in two mechanistically unrelated models, depleted osteoblasts and HSC niche function causing robust HSPC mobilization into the blood. These data demonstrate that BM macrophages play a critical role in the maintenance of endosteal HSC niches in vivo.

Since osteoblasts do not express G-CSFR, the mechanisms of G-CSF-mediated osteoblast suppression must be indirect16,23, and a number of mediators have been proposed. Although adrenergic receptors are involved in HSPC mobilization in response to G-CSF16,
they are clearly not the only players. In mice knocked-out for both β2 and β3 adrenergic receptors, HSPC mobilization in response to G-CSF was reduced by only 50%\textsuperscript{44}. Furthermore, treatment with the β adrenergic antagonist propanolol in WT adult mice reduced G-CSF-induced HSPC mobilization by only 30%\textsuperscript{16}. BM leukocytes also play an important role in osteoblast regulation. Indeed in G-CSFR KO to WT transplantation chimeras, where nerve and BM stromal cells express G-CSFR but donor BM leukocytes do not, CFC are no longer mobilized in response to G-CSF nor are osteoblasts suppressed\textsuperscript{17}. Therefore BM leukocytes are critical to both endosteal osteoblast suppression and HSPC mobilization.

Osteoclasts have also been reported to play a role in HSPC mobilization by secreting cathepsin K, which cleaves and inactivates CXCL12 and KL\textsuperscript{45}. These conclusions were drawn from experiments at a single time point of G-CSF mobilization. However, our data clearly demonstrate that neither osteoclast number nor cathepsin K transcription is increased during the initiation of G-CSF-mediated HSC mobilization. In fact a rise in osteoclasts and cathepsin K occurs subsequently when mobilization is over (herein and ref\textsuperscript{14}). Furthermore, osteoclast inhibition with the potent bisphosphonate zoleodronate did not inhibit, but enhanced mobilization of CFC and competitive repopulating cells. We now have congruent data from two inbred mouse strains demonstrating that i) osteoclast numbers and bone degradation increase only after cessation of G-CSF treatment and ii) that bisphosphonates (zoleodronate or pamidronate\textsuperscript{15}) synergize with G-CSF to increase CFC mobilization. Additionally in the two macrophage-depletion models used here, osteoclast numbers were reduced as a result of the depletion strategies, yet robust HSC mobilization occurred. Hence, we conclude that osteoclasts and release of cathepsin K are not the prime initiators of osteoblast suppression and HSC mobilization in response to G-CSF.

Tissue macrophages are phenotypically and functionally diverse, some sub-populations have trophic roles specific to their resident tissue, particularly in tissue development\textsuperscript{46} and wound healing\textsuperscript{47}. BM contains at least two trophic resident macrophage populations: central BM macrophages and endosteal osteomacs\textsuperscript{26}. Central BM macrophages are located throughout the BM stroma and often cluster with erythroid precursors to support erythropoiesis\textsuperscript{48}. More recently we described osteomacs, which support osteoblast function and maintain bone forming surfaces \textit{in vivo}\textsuperscript{26}. We now provide evidence that BM macrophage populations are also essential for the maintenance of the endosteal HSC niche. Furthermore, their depletion results in loss of endosteal osteoblasts, down-regulation of CXCL12, KL and Ang-1 mRNA at the endosteum, and mobilization of HSPC into the blood.
Following administration of G-CSF we find osteomacs vacate endosteal surfaces as early as day 1-2 after treatment, paralleling or even preceding endosteal osteoblast depletion. This sequence of events observed in mice administered G-CSF almost exactly phenocopies that observed during macrophage-depletion by either ligand-treated Mafia transgenic mice, or clo-lip administration in wild-type mice. In particular during clo-lip treatment, disappearance of osteomacs at endosteal bone surfaces and of over 95% of F4/80^+Ly-6G^+CD11b^+ phagocytic macrophages coincided with initiation of HSPC mobilization into blood. At this time endosteal osteoblasts persisted on endosteal surfaces although a marked drop in mRNA for HSC trophic cytokines confirmed that osteoblast function was already compromised. Of note, the chain of events following clo-lip administration was not dependent on mature granulocytes, or on the release of proteases NE, catG or MMP-9 in the BM. Indeed Clo-lip induced HSPC mobilization, osteomac loss and inhibition of osteoblast function even in GCSFR KO mice, despite few mature granulocytes and extremely low protease levels in the BM. Furthermore, in the mafia model, granulocytes and macrophages were both ablated, but HSPC were still mobilized. Therefore, granulocytes and their proteases are unlikely to be the primary drivers of osteoblast depletion and HSPC mobilization following macrophage depletion. This does not exclude the possibility that granulocytes subsequently amplify the mobilization response as suggested by increased mobilization at day 4 of clo-lip treatment when F4/80^+Ly-6G^+CD11b^+ granulocytes accumulate in the BM. Our data are consistent with a recent abstract showing that activation of G-CSF signaling in CD68-expressing monocytes/macrophages alone in GCSFR KO mice which are neutropenic, is sufficient to elicit HSPC mobilization^{49}.

In conclusion we show that BM macrophages are required for the maintenance of HSC niches. Loss of these macrophages initiates a cellular cascade that ultimately collapses this trophic niche environment through loss of endosteal osteoblasts and subsequently egress of HSPC into the blood. While anatomic location of osteomacs within the endosteal niche supports the notion that this population of BM macrophages are HSC niche supportive, definitive dissection of the role of osteomacs versus central BM phagocytic macrophages is required. The mechanisms by which macrophages regulate HSC niches and whether they themselves directly interact with HSC are currently under investigation.
ACKNOWLEDGEMENTS
The authors want to thank R Wadley for the cell sorting and Dr L Purton and Pr TJ Martin for insightful discussions. This work was supported by grants 288701, 434515 (JPL and IGW), 455941 (ARP and LJR) and 345401 (NAS) from the National Health and Medical Research Council, Australia (NHMRC). IGW, NAS, ARP and FH are Research Fellows of the NHMRC. JPL is a Senior Research Fellow of the Cancer Council of Queensland.

AUTHORSHIP
Contribution: I.G.W., N.A.S., and A.R.P. planned and performed experiments, interpreted results and wrote and edited the manuscript. V.B., B.N., F.H., I.J.P. and K.A.A. performed experiments and analyzed data. N.V.R. provided essential reagents and expertise. L.J.R. co-conceived the work, planned and performed experiments, interpreted results and wrote and edited the manuscript. J.P.L. conceived and coordinated the work, planned and performed experiments, interpreted results and wrote and edited the manuscript.

Conflict of interest disclosure: All the authors declare no competing financial interests.
References


Table 1. Primers and probes used in RT-qPCR analyses

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<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe Sequence</th>
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<td>osteocalcin</td>
<td>forward (32f) 5’-TTCTGCTCACTCTGCTGACCCT-3’</td>
<td>reverse (137b) 5’-CCCTCCTGGCTTTGGAATGAA-3’</td>
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<tr>
<td>osterix</td>
<td>forward (126f) 5’-AGCTCACTATGGCTCCAGTCC-3’</td>
<td>reverse (245b) 5’-GCGATGGCTTTGGAATGAA-3’</td>
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<tr>
<td>CXCL12</td>
<td>forward (168f) 5’-ATGCCCCTGCGGTTCT-3’</td>
<td>reverse (235b) 5’-TGTTGAGGATTTTCAGATGCTTG-3’</td>
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<td>KL</td>
<td>forward (80f) 5’-GCTACCCAATGCTGGGACTA-3’</td>
<td>reverse (286b) 5’-CCGAGATCTCCCTGGTTT-3’</td>
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<tr>
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<td>reverse (1806b) 5’-ATGGTGAGGAAAGTAAAGGA-3’</td>
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<td>VE-cadherin</td>
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<td>reverse (902b) 5’-TGGGTCACAAAACAGTCAGAA-3’</td>
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<td>fibronectin</td>
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<td>PTHR1</td>
<td>forward (894f) 5’-GATTCTGGTGAGGGACTG-3’</td>
<td>reverse (1090b) 5’-GGATGATCCACTTCTTGTGC-3’</td>
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<td>β2-microglobulin</td>
<td>Forward 5’-TTCCACCCACTGAGACTGT-3’</td>
<td>reverse 5’-GTCTTGCGCTCGCCAT-3’</td>
<td>probe (94f) FAM-5’-CAGTGACCCGTATGCATCTCA-3’-BHQ1</td>
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Table 2: GFP expression in stromal cells, myeloid cells, hematopoietic progenitor and stem cells from Mafia mice

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<th>Phenotypes</th>
<th>% GFP&lt;sup&gt;+&lt;/sup&gt; cells</th>
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<tr>
<td>Myeloid cells</td>
<td>CD11b&lt;sup&gt;-&lt;/sup&gt;</td>
<td>88.4 ± 0.7</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>CD11b&lt;sup&gt;+&lt;/sup&gt; F4/80&lt;sup&gt;-&lt;/sup&gt; Ly-6G&lt;sup&gt;+&lt;/sup&gt;</td>
<td>96.9 ± 0.3</td>
</tr>
<tr>
<td>Macrophages</td>
<td>CD11b&lt;sup&gt;+&lt;/sup&gt; F4/80&lt;sup&gt;-&lt;/sup&gt; Ly-6G&lt;sup&gt;-&lt;/sup&gt;</td>
<td>85.5 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>CD11b&lt;sup&gt;+&lt;/sup&gt; F4/80&lt;sup&gt;+&lt;/sup&gt; Ly-6G&lt;sup&gt;+&lt;/sup&gt;</td>
<td>88.6 ± 0.7</td>
</tr>
<tr>
<td>Endothelial</td>
<td>Lin&lt;sup&gt;-&lt;/sup&gt; CD45&lt;sup&gt;-&lt;/sup&gt; CD31&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.1 ± 0.1</td>
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<tr>
<td>MSC</td>
<td>Lin&lt;sup&gt;-&lt;/sup&gt; CD45&lt;sup&gt;-&lt;/sup&gt; CD31&lt;sup&gt;-&lt;/sup&gt; Sca1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Osteoblasts</td>
<td>Lin&lt;sup&gt;-&lt;/sup&gt; CD45&lt;sup&gt;-&lt;/sup&gt; CD31&lt;sup&gt;-&lt;/sup&gt; Sca1&lt;sup&gt;-&lt;/sup&gt; CD51&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.6 ± 0.1</td>
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</table>

Data are average ± SD of 3 mice. Wild-type mice had no cells (less than 0.05%) expressing GFP.
**Figure Legends**

**Figure 1. Impaired endosteal bone formation and HSC niche function in G-CSF-mobilized mice.** (A) Mobilization of CFC, Lin*Sca1*KIT+ (LSK) HSPC and Lin*CD41*Sca1*KIT*CD48*CD150+ phenotypic HSC in blood following G-CSF administration. (B) Histomorphometry for osteoblast surface per unit of bone surface (ObS/BS) and osteoid surface per bone surface (OS/BS) in the trabeculae of the proximal tibial secondary spongiosa from mice mobilized for 6 days with G-CSF. Data are mean ± SEM of both tibias from 3 to 6 mice per group from one representative experiment of two independent experiments. (C) Dynamic histomorphometry in mice mobilized with G-CSF. Mice treated with saline or G-CSF for 6 days were injected every third day alternatively with either calcein or xylenol orange and sacrificed at day 12. Tibial sections were analyzed by confocal laser scanning micrographs (x 20). Green and red lines are areas of bone that mineralized and incorporated calcein and xylenol orange respectively. BM is slightly red due to non-specific incorporation of xylenol orange by BM cells whereas solid bone is dark except where labeled. Top micrographs are across the cortical bone on the anterofibular side whereas bottom micrographs are across the secondary spongiosa of the metaphysis. (D) The incorporation of calcein lines was quantified in the secondary spongiosa and at the periosteum; shown are percentage of trabecular bone surface covered by double calcein lines (dLS/BS), trabecular mineral appositional rate (MAR) between double calcein lines, trabecular bone forming rate corrected for bone surface (BFR/BS), and periosteal mineral appositional (PsMAR). (E) Expression levels of CXCL-12, KL and Ang-1 mRNA in endothelial cells ( ), mesenchymal stromal cells (■) and osteoblasts (■) sorted from non-mobilized endosteum, and whole BM cells ( ). All expression levels are relative to β2-microglobulin mRNA and are the average of two experiments in which populations were sorted from ten mice each. (F) mRNA for CXCL-12, KL, Ang-1 and fibronectin were quantified at the indicated time-points of G-CSF-mobilization by RT-qPCR. All expression levels are relative to β2-microglobulin mRNA. Each symbol represents the result from an individual mouse. Data are means ± SD of 4 mice per group. *** = p<0.001, ** = p< 0.01, and * = p < 0.05.

**Figure 2. G-CSF induced mobilization is not mediated by osteoclasts.** (A) TRAP staining in tibial spongiosa from mice treated with saline (Sal) or G-CSF for 2 days (G2), 4 days (G4) or 6 days followed by 4 days of recovery (G10). Osteoclasts are stained in red. B: Histomorphometry for osteoclast surface per bone surface (OcS/BS) and number of
osteoclasts per bone perimeter (NOc/BPm) in the trabeculae of the proximal tibial secondary spongiosa from mice mobilized for 6 days with G-CSF. C: RT-qPCR for cathepsin K mRNA in endosteal cells. * indicates p < 0.05. D-F: Effect of zoledronic acid and G-CSF treatment on osteoclast (D) and osteoblast (E) surface per bone surface in the trabeculae, mobilization of CFC into blood (F) and spleen (G), and mobilization of competitive repopulating cells (H). In panels B, D-E, each bar is mean ± SEM of 4 mice per group. In panels C, F-H, each symbol represents an individual mouse and bars are the mean from one representative experiment out of 2 independent experiments.

**Figure 3. Loss of osteomacs from endosteal surface during G-CSF-induced mobilization.** Bone sections from mice treated with saline (A,B), or G-CSF for 2 days (C), 6 days (D) or for 6 days with G-CSF following by a recovery of 2 days (E) or 4 days (F) were stained with a monoclonal antibody against the F4/80 antigen (B-F) or an isotype-matched non immune antibody (A). F4/80+ cells are stained brown. F4/80+ osteomacs forming a canopy over active osteoblasts at endosteal surfaces were detected in saline treated animals (B, arrows) as well as 4 days following cessation of a 6 day G-CSF treatment (F). Clustering of F4/80+ cells around blood vessels was observed after 2 days of treatment (C, arrows). Pictures are representative of one mouse out of 3 mice per time-point.

**Figure 4. In vivo depletion of myeloid cells in Mafia mice results in HSPC mobilization and collapse of endosteal niches.** Effect of a 5 day systemic treatment with AP20187 ligand (filled columns) or vehicle (empty columns) in Mafia and wild-type mice on femoral content in CD11b+GFP+ myeloid cells (A) and CD11b+Ly6-G+ granulocytes (B), number of CFC mobilized into blood (C) and spleen (D). CD11b+GFP+ myeloid cells were not determined (ND) in wild-type mice as they do not express the GFP transgene. Data are mean ± SD of 4-6 mice per group. *** = p<0.001, ** = p< 0.01, and * = p < 0.05 between vehicle and AP20187 treated mice. (E) Effect of a 5 day systemic treatment with AP20187 ligand (filled symbols) or vehicle (empty columns) in Mafia and wild-type mice on osteocalcin, Ang-1 and KL in endosteal cells measured by RT-qPCR. Each symbol represents an individual mouse, bars are means. (F) Immunohistochemical staining of tibial sections from Mafia and C57BL/6 mice treated for 5 days with AP20187 or vehicle. Brown positive staining for F4/80 (left panels) or osteocalcin (right panels) is shown in near serial section of metaphyseal and diaphyseal endocortical regions. F4/80+ osteomacs can be seen directly on bone surfaces or immediately adjacent to cuboidal osteocalcin+ osteoblasts in control groups and osteomac canopy structure.
is clearly evident on endocortical bone. Dramatic depletion of F4/80$^+$ cells and subsequent loss of osteocalcin$^+$ osteoblasts is clearly evident in ligand treated Mafia mice. Original magnification 20x and 40x. All sections were counterstained with hematoxylin.

**Figure 5. In vivo depletion of macrophages with clodronate loaded liposomes mobilizes HSC.** (A) Effect of a 2 day treatment with clo-lip and PBS-lip on the number of BM F4/80$^+$Ly-6G$^-$$^-$CD11b$^+$ (F+G$-$), F4/80$^+$Ly-6G$^+$CD11b$^+$ (F+G$^+$) macrophages and F4/80$^-$Ly-6G$^+$CD11b$^+$ (F-G$^+$) granulocytes measured by flow cytometry. The two dot-plots illustrate the loss of F+G$. Data are mean ± SD of 4 mice per group. (B) Effect of liposomes on the number of circulating Lin$^-$$^-$Sca1$^+$KIT$^+$ HSPC and CFC. Data are mean ± SD of 4 mice per group. (C) Mobilization of long-term reconstituting HSC in competitive repopulation assay. Test donor CD45.2$^+$ mice were injected with clo-lip for 2 or 4 days, or PBS-lip for 4 days. 50µL blood was transplanted in competition with 200,000 competing CD45.1$^+$ BM cells into lethally irradiated CD45.1$^+$ recipients. Donor CD45.2$^+$ contribution was measured in CD45$^+$ leukocytes, myeloid, B and T cells 16 weeks after transplantation. Contribution was estimated to be positive when proportion of CD45.2$^+$ donor cells was above 0.5% in each lineage. *** = p<0.001, ** = p< 0.01, and * = p < 0.05 between PSB-lip and clo-lip treated mice.

**Figure 6. Effect of clodronate loaded liposomes on endosteal niches.** (A) Time-course of clo-lipo treatment on mRNA expression of osteocalcin, CXCL12, KL and Ang-1 in endosteal cells relative to β2-microglobulin. Each symbol represents one mouse and bars are average for each group. *** = p<0.001, ** = p< 0.01, and * = p < 0.05 between PSB-lip (time 0) and clo-lip treated mice. (B) Immunohistochemical staining of femoral sections from C57BL/6 mice treated with clo-lip (24 or 48hrs) or PBS-lip (48hrs). Brown positive staining for F4/80 and osteocalcin is shown in near serial sections of metaphysal bone. Clo-lip treatment resulted in depletion of F4/80$^+$ cells at 24 and 48hrs post clo-lip treatment. Subsequent reduction in osteocalcin$^+$ osteoblasts occurred at 48hrs. All sections were counterstained with hematoxylin. * = p<0.05; ** = p< 0.01; *** = p<0.001.
**Figure 7. Mobilization with clo-lip is independent of neutrophil proteases.** (A) Concentration of proteolytically active NE and catG in BM fluids from clo-lip treated C57BL/6 mice. Results are compared to 129Sv mice treated for 4 days with G-CSF (4 mice per time-point, mean±SD). (B) Gelatin zymography of BM fluids from C57BL/6 mice treated with clo-lip or G-CSF. Each lane contains a pool of BM fluids from 4 different mice. (C) Effect of G-CSF on osteoid surface per bone surface (OS/BS), osteoblast surface per bone surface (ObS/BS), number of osteoblasts per bone perimeter (NOb/BPm) in DPP1-deficient mice. Data are means±SEM of both tibias from 4 mice per group. Effect of clo-lip in GCSFR-deficient mice on mobilization of CFC in blood (D), and (E) concentrations of active NE, catG and MMP-9 in BM fluids (4 mice per time-point, mean±SD). Effect of 4 day clo-lip treatment on osteoid surface per bone surface (OS/BS), osteoblast surface per bone surface (ObS/BS), number of osteoblasts per bone perimeter (NOb/BPm) (F), osteocalcin+ mature osteoblasts (G, right panels, metaphyseal trabecular bone) and F4/80+ osteomacs (G, left panels) in GCSFR KO mice (means±SEM and representative 40x magnification images respectively, 3 mice per group). * = p<0.05; ** = p<0.01; *** = p<0.001.
Figure 1
Figure 2
Figure 3
Figure 4
**Figure 5**

**A** Gated on CD11b+ myelocytes

<table>
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<tr>
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<th>PBS-lip 2 days</th>
<th>Clo-lip 2 days</th>
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<tr>
<td>F+G-</td>
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<tr>
<td>F+G+</td>
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<td>Ly6-G</td>
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**B**

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<td>3</td>
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<tr>
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**C**

Liposomes i.v.  
Blood 50µL  
11.0 Gray  
Primary recipient CD45.1  
16 weeks  
2x10^5 BM cells  

**CD45+ leukocytes**  
**CD11b+ myeloid cells**  
**B220+ B cells**  
**CD3+ T cells**

**PBS-lip**  
**Clo-lip**  
4 days  
2 days  
4 days  
4 days

***  
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****  
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Figure 6
Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSC


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