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Osteoclasts are Dispensable for Hematopoietic Progenitor Mobilization by G-CSF in Mice

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Abstract

The contribution of osteoclasts to hematopoietic stem/progenitor cell (HSPC) retention in the bone marrow is controversial. Studies of HSPC trafficking in osteoclast-deficient mice are limited by osteopetrosis. Here, we employed two non-osteopetrotic mouse models to assess the contribution of osteoclasts to basal and granulocyte colony-stimulating factor (G-CSF) induced HSPC mobilization. We generated *Rank*^{-/-} fetal liver chimeras using *Csf3r*^{-/-} recipients to produce mice lacking G-CSF receptor expression in osteoclasts. Basal and G-CSF-induced HSPC mobilization was normal in these chimeras. We next acutely depleted osteoclasts in wild-type mice using the RANK ligand inhibitor osteoprotegerin (OPG). Marked suppression of osteoclasts was observed after a single injection of OPG-Fc. Basal and G-CSF-induced HSPC mobilization in OPG-Fc treated mice were comparable to control mice. Together, these data show that osteoclasts are not required for the efficient retention of HSPCs in the bone marrow and are dispensable for HSPC mobilization by G-CSF.

INTRODUCTION

Granulocyte-colony stimulating factor (G-CSF) is the most commonly used agent to mobilize hematopoietic stem/progenitor cells (HSPCs) from the bone marrow to blood. G-CSF induces HSPC mobilization, in large part, by suppressing CXCL12 production from bone marrow stromal cells.^{1–4} We recently reported that expression of the G-CSFR only in CD68+ monocytic-lineage cells is sufficient to induce HSPC mobilization by G-CSF.⁵ Within the monocytic lineage, the G-CSFR is expressed on monocytes, macrophage, myeloid dendritic cells, and osteoclasts. There is evidence linking macrophages with HSPC trafficking. Specifically, pharmacologic or genetic approaches that delete macrophages

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Contribution: MR, TS, and APS participated in the experimental design, performed the experiments, analyzed the data, and participated in writing the manuscript. DCL participated in the experimental design, data interpretation, and assisted with the writing of the manuscript.

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result in suppression of CXCL12 production from stromal cells and HSPC mobilization.^{6,7} However, the contribution of osteoclasts to HSPC mobilization is less clear. Kollet and colleagues reported that activation of osteoclasts by injection of RANK ligand (RANKL) is associated with moderate HSPC mobilization.⁸ Likewise, Cho et al showed that activation of osteoclasts with RANKL enhanced mobilization in response to inflammatory stress.⁹ These data suggest that osteoclasts promote HSPC egress from the bone marrow. Conversely, there is data suggesting that osteoclasts may contribute to HSPC retention in the bone marrow. Inhibition of osteoclast activity with bisphosphonate treatment is associated with a modest increase in HSPC mobilization by G-CSF.^{7,10} Moreover, Miyamoto and colleagues showed that in several transgenic mouse lines with impaired osteoclast activity (specifically, *Csf1*, *Fos*, or *Rankl* deficient mice) G-CSF-induced HSPC mobilization is increased.¹⁰ Each of these studies has certain limitations. Most notably, osteoclast deficiency is associated with osteopetrosis, extramedullary hematopoiesis, and constitutive HSPC mobilization, potentially confounding studies of HSPC trafficking. Here, we employed two non-osteopetrotic mouse models to assess the contribution of osteoclasts to basal and G-CSF induced HSPC mobilization.

METHODS

Mice

Sex- and age-matched wild-type, *Csf3r*^{-/-} mice¹¹ and *Rank*^{+/-} mice¹² on a C57BL/6 background were maintained under standard pathogen free conditions according to methods approved by the Washington University Animal Studies Committee.

Rank^{-/-} fetal liver chimeras

Fetal liver cells were harvested from a *Rank*^{+/-} mouse intercross at 13.5–14.5 days post-conception. Genotyping was performed on fetal tissue to identify *Rank*^{-/-} fetal livers.¹² Two million cells were injected retro-orbitally into lethally irradiated (1,100 cGy) wild-type or *Csf3r*^{-/-} 6–12 week old mice.

G-CSF and OPG-Fc

6–8 week old male wild-type mice were used in these experiments. Recombinant human G-CSF (Amgen) was administered at a dose of 250 µg/kg/day subcutaneously for 7 days. Mice were analyzed 3 to 4 hours after the final cytokine dose. Recombinant OPG-Fc was a generous gift from Amgen. Mice were given a single subcutaneous injection of 100 µg of OPG-Fc or saline alone.

Colony-forming cells (CFU-C) assays

We plated 10 µL blood, 5×10^4 nucleated spleen cells, or 2.0×10^4 nucleated bone marrow cells in 1.25 mL methylcellulose media supplemented with a cocktail of recombinant cytokines (MethoCult 3434; StemCell Technologies). Colonies were counted after 7 days of culture in a humidified chamber with 5% carbon dioxide (CO₂) at 37°C.

Real-time quantitative RT-PCR

Femurs were flushed directly with TRIzol reagent (Invitrogen), and RNA was isolated according to the manufacturer's instructions. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed using the TaqMan One-step RT-PCR Master Mix Reagents Kit on a GeneAmp 5700 Sequence Detection System (Applied Biosystems). TaqMan primer/probe mixes for cathepsin K (Mm00484039_m1), tartrate-resistant acid phosphatase (Mm00475698_m1), *Csf3r* (Mm00432735_m1), and beta-actin (Mm00607939_s1) were obtained from Applied Biosystems.

Flow Cytometry

The following antibodies were used (all from eBiosciences): Gr-1 (RB6-8C5), B220 (RA3-6B2), CD3e (145-2C11), and Ter-119 (TER-119); Sca-1 (D7); and c-kit (2B8). Cells were analyzed either on a FACScan or Gallios flow cytometer.

TRAP staining

Paraffin-embedded bone marrow sections were stained for TRAP and quantified by histomorphometry, as described previously.¹³

Statistics

Statistical significance of differences was calculated using 2-tailed Student's t tests.

RESULTS & DISCUSSION

To generate adult mice with defective osteoclasts but without severe osteopetrosis, we first generated *Rank*^{-/-} fetal liver chimeras (Figure 1). RANK signaling is required for osteoclast development;¹² thus, we predicted that *Rank*^{-/-} fetal liver chimeras would lack osteoclasts. However, osteoclasts number was only modestly reduced in these mice (Figure 1A–D). This is likely secondary to radio-resistant recipient osteoclasts and is consistent with a recent report showing recipient macrophage expansion following transplantation, if donor macrophage development is compromised.¹⁴

To circumvent this problem, we transplanted *Rank*^{-/-} (or *Rank*^{+/+}) fetal liver cells into *Csf3r*^{-/-} recipients (Figure 1E). Since donor osteoclast development is blocked in the absence of RANK signaling, any remaining osteoclasts in these mice should lack the G-CSF receptor. Donor engraftment with *Csf3r* sufficient cells was confirmed by measuring *Csf3r* mRNA expression in bone marrow cells 6–8 weeks after transplantation (Figure 1F). Of note, the number of osteoclasts (as measured by bone marrow cathepsin K and TRAP expression) was comparable in *Csf3r*^{-/-} recipients reconstituted with wild-type or *Rank*^{-/-} bone marrow (Figure 1G & H). As expected, these mice are not osteopetrotic, as evidence by bone marrow histology (Suppl. Figure 1) and normal bone marrow and spleen cellularity (Suppl. Figure 2). At baseline, the number of bone marrow, peripheral blood, and spleen Kit⁺ Sca⁺ lineage⁻ (KLS) cells and colony forming cells (CFU-C) were similar in *Rank*^{-/-} *Csf3r*^{-/-} and *Rank*^{+/+} *Csf3r*^{-/-} chimeras (Figure 1I & J). Moreover, a similar robust mobilization response to G-CSF with both types of chimeras was observed.

The *Rank*^{-/-} *Csf3r*^{-/-} chimera data show that G-CSF signaling in osteoclasts is not required for HSPC mobilization by G-CSF. However, it is possible that osteoclasts may regulate HSPC trafficking independent of the G-CSF receptor. To address this possibility, we acutely depleted osteoclasts by treating wild-type mice with OPG-Fc. Osteoprotegerin (OPG) is a decoy receptor for RANKL.¹⁵ Recombinant OPG-Fc is a chimeric molecule fusing the RANKL binding domain of OPG with the Fc portion of human immunoglobulin. OPG-Fc has a long circulating half-life, and treatment with OPG-Fc reversibly depletes osteoclasts in rats.¹⁶ As expected, we observed a marked suppression of osteoclasts in wild-type mice after a single injection of OPG-Fc that persisted for at least 9 days. Cathepsin K and tartrate resistant acid phosphatase (TRAP) mRNA expression in the bone marrow decreased 50-fold and 8.4-fold, respectively (Figure 2A & B), and TRAP⁺ cells were nearly absent [Figure 2C & D and Suppl. Figure 3; number of osteoclasts per mm bone surface: 3.5 ± 0.11 (saline treated) versus $0.19 \pm .02$ (day 7 after OPG-Fc treatment), $P < 0.001$]. Of note, OPG-Fc had no effect on monocytes, macrophages, and myeloid dendritic cell number (Suppl. Figure 4). Osteoclast depletion by itself did not result in mobilization of KLS cells to blood or spleen (Figure 2E). We next treated mice with OPG-Fc followed by G-CSF (Figure 2F). Again, severe osteoclast suppression was observed in OPG-Fc treated mice, whether or not G-CSF also was given (Figure 2G). Importantly, G-CSF induced KLS cell and CFU-C mobilization to blood and spleen was similar in OPG-Fc treated and control mice (Figure 2H & I).

Collectively, our data show that osteoclasts are not required for the efficient retention of HSPCs in the bone marrow and are dispensable for HSPC mobilization by G-CSF. Consequently, the increase in basal and G-CSF induced HSPC mobilization observed in constitutive models of osteoclast deficiency are likely secondary to osteopetrosis. Of note, treatment with bisphosphonates augments HSPC mobilization by G-CSF in mice,^{7,10} suggesting that off-target effects of these agents, rather than osteoclast suppression, is responsible for the enhanced mobilization response. Collectively, these data suggest that pharmacologic strategies to augment HSPC mobilization by disrupting osteoclast function are unlikely to be effective.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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KEYPOINTS

1. Osteoclasts are dispensable for HSPC retention in the bone marrow
2. Osteoclasts are not required for G-CSF induced HSPC mobilization

Figure 1. G-CSF signaling in osteoclasts is dispensable for HSPC mobilization by G-CSF
Fetal liver cells from either *Rank*^{+/+} or *Rank*^{-/-} mice were transplanted into lethally-irradiated mice. (A & B) Shown are representative bone sections from *Rank*^{+/+} (A) and *Rank*^{-/-} (B) chimeras stained for tartrate-resistant acid phosphatase (TRAP), a marker of osteoclasts. (C & D) The degree of osteoclast loss in the mice was quantified by measuring bone marrow cathepsin K mRNA (C) or TRAP mRNA (D) expression relative to β -actin mRNA (n = 2–4 mice per genotype). (E) Fetal liver cells from either *Rank*^{+/+} or *Rank*^{-/-} mice were transplanted into lethally-irradiated *Csf3r*^{-/-} mice. Eight weeks later, mice were

treated with either PBS or 250 µg/kg/day G-CSF for 7 days. Shown is the expression of *Csf3r* (**F**) Cathepsin K (**G**) or TRAP (**H**) mRNA in the bone marrow of wild type mice (WT), *Csf3r*^{-/-} mice, or untreated *Rank*^{+/+} or *Rank*^{-/-} *Csf3r*^{-/-} chimeras relative to β-actin mRNA (n = 4–6 per cohort). (**I & J**) The number of KLS cells (**I**) or CFU-Cs (**J**) in the peripheral blood, bone marrow, and spleen (n = 4–6 mice per donor genotype per treatment group) is shown. *P < 0.05, **P < 0.01, ***P < 0.001, ns: not significant, nd: not detected.

Figure 2. Osteoclast ablation does not perturb basal or G-CSF-induced HSPC mobilization
(A & B) Mice were treated with a single dose of OPG-Fc at day 0. Osteoclast number was assessed by measuring bone marrow cathepsin K **(A)** or TRAP **(B)** mRNA expression relative to β -actin mRNA ($n = 2-3$ per time point). **(C & D)** Representative sections of trabecular bone stained for TRAP from untreated **(C)** or OPG-Fc treated **(D)** mice 7 days after treatment with a single dose of OPG-Fc. **(E)** Wild-type mice were given a single dose of OPG-Fc at day 0; the frequency of KLS cells in the peripheral blood, bone marrow, and spleen was assayed at the indicated time points. The last bar on each graph is based on mice

treated with G-CSF alone for 5 days ($n = 2-5$ per time point). (**F**) Mice were treated with either a single dose of OPG-Fc, a five-day course of G-CSF beginning 3 days after the OPG-Fc treatment, or a 5-day course of G-CSF alone. (**G**) Cathepsin K or TRAP mRNA levels in the bone marrow was measured on day 7. (**H & I**) The number KLS cells (**H**) or CFU-C (**I**) in the peripheral blood, bone marrow, and spleen was measured at the conclusion of the G-CSF course ($n = 2-5$ per time point). ns: not significant.