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Altered Hematopoietic Stem Cell and Osteoclast Precursor Frequency in Cathepsin K Null Mice

Christian Jacome-Galarza,[1,2] Do Yu Soung,[1,3] Naga Suresh Adapala,[1,3] Maureen Pickarski,[4] Archana Sanjay,[1,3] Le T. Duong,[4] Joseph A. Lorenzo,[1,2**] and Hicham Drissi[1,2*]

[1] *New England Musculoskeletal Institute, University of Connecticut Health Center, Farmington, Connecticut 06030*
[2] *Department of Medicine, University of Connecticut Health Center, Farmington, Connecticut 06030*
[3] *Department of Orthopaedics, University of Connecticut Health Center, Farmington, Connecticut 06030*
[4] *Merck Sharp & Dohme Research Laboratories, West Point, Philadelphia 19486*

ABSTRACT

Cathepsin K (CatK) is a lysosomal cysteine protease necessary for bone resorption by osteoclasts (OCs), which originate from myeloid hematopoietic precursors. CatK-deficient ($CatK^{-/-}$) mice show osteopetrosis due to defective resorption by OCs, which are increased in number in these mice. We investigated whether genetic ablation of CatK altered the number of hematopoietic stem cells (HSCs) and OC precursor cells (OCPs) using two mouse models: $CatK^{-/-}$ mice and a knock-in mouse model in which the CatK gene (*ctsk*) is replaced by *cre* recombinase. We found that CatK deletion in mice significantly increased the number of HSCs in the spleen and decreased their number in bone marrow. In contrast, the number of early OCPs was unchanged in the bone marrow. However, the number of committed $CD11b^{+}$ OCPs was increased in the bone marrow of $CatK^{-/-}$ compared to wild-type (WT) mice. In addition, the percentage but not the number of OCPs was decreased in the spleen of $CatK^{-/-}$ mice relative to WT. To understand whether increased commitment to OC lineage in $CatK^{-/-}$ mice is influenced by the bone marrow microenvironment, $CatK^{Cre/+}$ or $CatK^{Cre/Cre}$ red fluorescently labeled OCPs were injected into WT mice, which were also subjected to a mid-diaphyseal femoral fracture. The number of OCs derived from the intravenously injected $CatK^{Cre/Cre}$ OCPs was lower in the fracture callus compared to mice injected with $CatK^{+/Cre}$ OCPs. Hence, in addition to its other effects, the absence of CatK in OCP limits their ability to engraft in a repairing fracture callus compared to WT OCP. J. Cell. Biochem. 115: 1449–1457, 2014.

KEY WORDS: CATHEPSIN K; HEMATOPOIESIS; OSTEOCLAST PRECURSORS; OSTEOPETROSIS

Osteoclasts (OCs) are specialized bone resorbing cells derived from monocyte/myeloid lineage in bone marrow [Teitelbaum, 2000]. The bone resorptive activity of OCs depends on several factors involving formation of ruffled membrane, acidification of resorptive surface and secretion of matrix degrading enzymes, of which is predominantly mediated by cathepsin K (CatK). This lysosomal cysteine protease CatK is active at acid pH and is highly expressed in OCs. Its function is to degrade the organic matrix of bone, particularly collagens, in the acidic environment of the sub-osteoclastic resorption space. In its absence, OC-mediated bone resorption is decreased, whereas bone formation is increased [Pennypacker et al., 2009]. Curiously, the bones of CatK-deficient mice demonstrate significantly increased number of non-resorbing OCs [Kiviranta et al., 2005], likely due to the increase in RANKL/OPG in the bone microenvironment [Lotinun et al., 2013].

Bone harbors marrow tissue, which contains several niches including the hematopoietic niche, which is populated with hematopoietic stem cells that give rise to all lineages of blood cells and undergo self-renewal. Alterations of the size of the bone marrow cavity due to osteopetrosis, as a result of defective OC-mediated resorption, influences hematopoiesis, and hematopoietic stem cell trafficking [Kollet et al., 2006; Kollet and Dar, 2007; Shivtiel

Christian Jacome-Galarza and Do Yu Soung contributed equally to this work.

*Correspondence to: Hicham Drissi, Department of Orthopedic Surgery, University of Connecticut Health Center, Farmington, CT 06030. E-mail: drissi@uchc.edu
**Correspondence to: Joseph A. Lorenzo, Department of Medicine, University of Connecticut Health Center, Farmington, CT 06030. E-mail: jlorenzo@NSO2.uchc.edu

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et al., 2008; Lymperi et al., 2011; Mansour et al., 2012; Li et al., 2013]. We investigated whether genetic deletion of cathepsin K can alter either the percentage or absolute number of hematopoietic stem cells and differentiated myeloid OC precursor (OCP) cells in the bone marrow, spleen, and peripheral blood of mice. We also examined the ability of OCP from CatK-deficient mice to migrate and engraft in bone at the site of a repairing fracture.

Two different mouse models of cathepsin K inhibition were investigated: (1) a gene-targeted deletion ($CatK^{-/-}$) that results in the absence of the protein, and (2) a knock-in mouse model in which the cathepsin K gene (*Ctsk*) is replaced by cre recombinase ($CatK^{cre/cre-}$). In both models, the bone resorbing activity of individual OCs is decreased, though the number of non-resorbing OCs on bone is significantly increased as previously described for $CatK^{-/-}$ mice [Pennypacker et al., 2009].

We analyzed both the percentage and absolute number of various hematopoietic lineage cells in the bone marrow, spleen, and peripheral blood of mice using flow cytometry and antibody expression paradigms that we previously defined [Jacquin et al., 2006; Jacome-Galarza et al., 2011, 2013]. As expected for mice with osteopetrosis, CatK deficient mice had a decrease in total marrow cellularity and the HSC pool in bone marrow. We also found an increase in the number of HSCs in the spleen, which was probably a compensation for the decrease in the number of HSCs in the bone marrow.

The percentage of early OCPs in bone marrow was not affected by CatK deficiency. However, the percentage of more mature myeloid lineage cells in bone marrow, expressing CD11b, which includes OCPs, was increased. In the spleen, the percentage but not the absolute number of OCPs was decreased while there was no difference in the percentage of OCPs in the peripheral blood. Finally, we found that the ability of CatK-deficient OCPs to migrate and engraft in the repairing fracture callus of WT mice was decreased relative to WT OCPs.

Together the above findings suggest that the absence of CatK promotes the differentiation of myeloid precursors toward the OC lineage. However, the bone marrow microenvironment that occurs with CatK loss of function may be required to promote osteoclastogenesis in vivo.

MATERIALS AND METHODS

MICE

Eight- to 10-week-old male wild-type and cathepsin K null mice [Pennypacker et al., 2009] bred into a C57BL6/L129 background were used to isolate bone marrow cells for the lineage analyses by FACS. For the fracture experiments, we generated $CatK^{Cre/+}$;tdTomato or $CatK^{Cre/Cre}$;tdTomato by crossing with tdTomato (B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$/J, Jax Laboratory, Bar Harbor, Main) and $CatK^{Cre/+}$ mice [Nakamura et al., 2007]. All mice were housed at the University of Connecticut Health Center (UCHC) animal facilities according to state and federal animal care guidelines. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Connecticut Health Center.

ANTIBODIES AND FLOW CYTOMETRY ANALYSIS

All the FACS specific antibodies used in this study for the phenotypic analysis of different cell populations are commercially available. Antibodies were either directly conjugated to specific fluorochromes or biotinylated and were from e-Biosciences (San Diego, CA), Biolegend (San Diego, CA), BD Bioscience/BD Pharmingen (San Diego, CA), or R&D Systems (Minneapolis, MN). The antibodies used in these studies were as follows: anti-progenitor cell antibodies: anti-Ly-6A/E Sca-1 (E13.161.7), anti-c-kit/CD117 (2B8), anti-c-fms/CD115 (AFS98), anti-adhesion molecules mAb: anti-Ly6C (AL-21), anti-Ly6G (1A8), anti-monocyte/macrophage antibodies: anti-CD11b/Mac-1 (M1/70), anti-F4/80 (BM8); anti-dendritic cell mAb: anti-CD11c (N418); anti-granulocyte mAb: anti-Ly-6G/Gr-1 (RB6-8C5), anti-B-cell lineage antibody (mAb): anti-CD45R/B220 (RA3-6B2); anti-T-cell lineage mAb: anti-CD3 (145-2C11); anti-NK cell mAb: NK1.1 (PK136). Labeling of cells with conjugated or biotinylated mAB followed by streptavidin conjugated fluorochrome was performed by standard staining procedures for flow cytometric analysis. The staining procedure was performed on ice, and dead cells were identified and excluded by propidium iodide (PI) staining. Flow cytometry analysis was performed in a BD-FACS LSR II (BD Biosciences, San Jose, CA). All the data were analyzed using FlowJo software from Tree Star, Inc. (Ashland, OR). The percentage of a specific population among live cells was multiplied by the total number of cells isolated from that tissue to calculate the absolute number of cells from that population in that tissue. The data were analyzed as the percentage of cells among live cells in the total population of cells detected by the flow cytometer.

PREPARATION OF BONE MARROW, SPLEEN, AND PERIPHERAL BLOOD FOR FLOW CYTOMETRY ANALYSIS

Bone marrow cells from the long bones of hind limbs, femora, and tibiae were flushed with 10 ml of staining medium (1× Hank's balanced salt solution [HBSS]; 10 mM HEPES; 2% newborn calf serum) into 15 ml tubes. Following centrifugation at 1,500 rpm for 5 min at 4°C, the cell pellet was resuspended and incubated for 5 min on ice in 1 ml of red blood cell lysis buffer (Sigma, St. Louis, MO). Cells were washed with staining medium and resuspended in 10 ml of staining medium and single-cell suspension was prepared by filtering through a 100-mm Nytex mesh. Live cells were counted in a hemocytometer using trypan blue exclusion. To obtain cells from spleen, cells were gently released using a pair of frosted microscope slides in 10 ml of staining media on a Petri dish. Following centrifugation, single-cell preparation was obtained by following the procedure described above for bone marrow cells. Peripheral blood was collected into 500 μl of 5 mM EDTA in PBS from the tail vein of mice, 2 ml of 2% dextran was added and the suspension was incubated at 37°C for 30 min. Single cell suspension of peripheral blood mononuclear cells was obtained from the cloudy upper phase by the method described above.

FRACTURE HEALING EXPERIMENTS

Animals were anesthetized and given analgesic prior to the surgical procedure. Femoral fractures were performed on the left limb of WT mice, as we previously described [Kaback et al., 2008a,b; Naik et al., 2009; Soung do et al., 2013]. Briefly, the surrounding area of

the left knee was shaved and prepared under sterile conditions. A 25-gauge needle was used to perforate the articular surface of the femoral condyle through the skin. This initial perforation was subsequently used to insert a precut stainless steel 316LVM wire (0.015 in. OD: SmallParts, Mirama, FL) into the medullary canal at the proximal end of the femur. The remaining end of the stylus was cut off and excess wire was bent and inserted under the skin. A mid-diaphyseal fracture was created using the Einhorn device [Kaback et al., 2008a,b; Naik et al., 2009; Soung do et al., 2013]. The fracture and the callus formation were radiographically monitored using a Faxitron X-ray (Wheeling, IL). Bone marrow cells were isolated from either Cathepsin K-TD tomato null mice or CatK$^{+/-}$ TD-tomato used as control. Fractured WT mice were intravenously injected with two million cells from either reporter genotype into the retro-orbital vein at the time of fracture and then re-injected with the same amount of cells ten days later. Animals were euthanized at day 18 post-fracture and fractured limbs were harvested for histological evaluations.

HISTOLOGICAL EVALUATIONS

Fractured limbs were harvested and the intramedullary pins carefully removed. Soft tissue excess was removed from the limbs and bones were fixed, decalcified, and embedded for histological analysis as described previously [Kaback et al., 2008b; Naik et al., 2009; Soung do et al., 2013]. Five-micron thick serial sections were stained with Kamiya TRAP staining kit (Kamiya Biomedical Company, Seattle, WA) following the manufacturer's instructions. Image acquisition was performed using a Q-Imaging Retig 200R camera connected to a Nikon Eclipse 50i microscope (Nikon, Melville, NY). For imaging of OCs within calluses, fractured bones fixed with 10% formalin were submerged with 30% sugar in PBS and were incubated at 4°C overnight. Bones were then embedded with O.C.T. compound (Tissue-Tek, Torrance, CA) for frozen tissue specimens. Seven-micron thick sections were used to detect TD-tomato positive cells within the calluses under fluorescent microscopy using a tetramethylrhodamine isothiocyanate (TRITC) filter.

STATISTICAL ANALYSIS

Statistical significance between different groups of mice was determined by unpaired Student's *t*-test using GraphPad Prism 5.

RESULTS

MICE LACKING CATHEPSIN K SHOW ALTERED NUMBERS OF HEMATOPOIETIC STEM CELLS IN THE BONE MARROW AND SPLEEN

Cathepsin K deficient (CatK$^{-/-}$) mice have defective bone resorption which results in osteopetrosis [Saftig et al., 1998]. Using 8-week-old male mice, we determined the effect of loss of CatK on the percentage and number of HSCs in the bone marrow and spleen. The total volume of the marrow cavity in CatK$^{-/-}$ mice is decreased due to osteopetrosis. Hence it was not surprising to find a decrease in the absolute number of cells in the bone marrow compared to WT mice (Fig. 1A). We also determined the total number of cells in the spleen to observe if the absence of CatK would affect other hematopoietic compartments. In contrast to the bone marrow, the spleens of CatK$^{-/-}$ mice showed a similar number of cells compared to the spleens of WT mice (Fig. 1B).

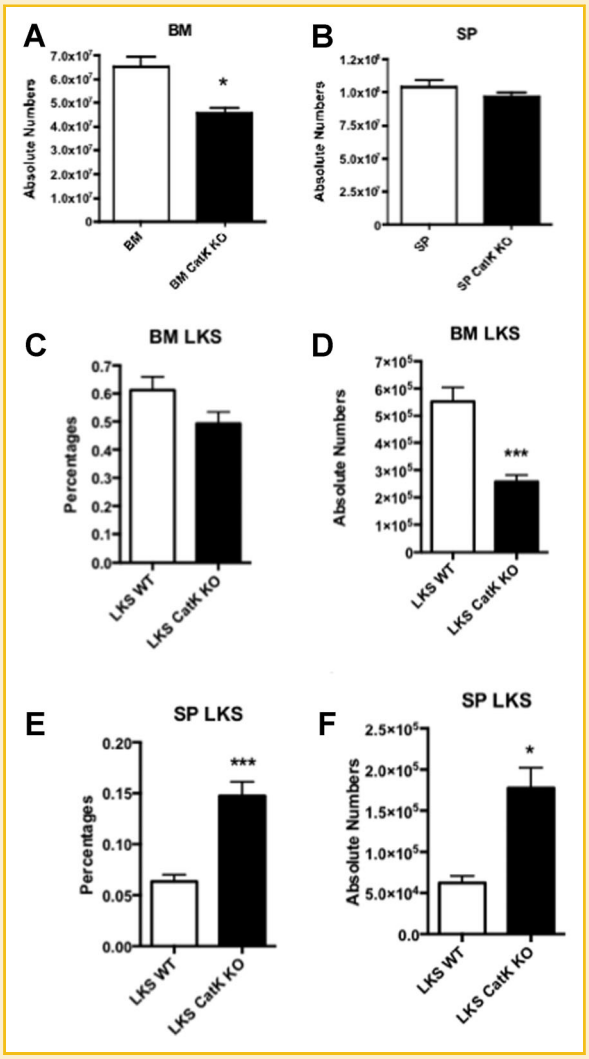

Fig. 1. Total cellularity and number of hematopoietic stem cells in the bone marrow and spleen of mice lacking cathepsin K. Total number of cells in the bone marrow and spleen of 8-week-old male mice was determined by cell counting method using Neubauer chamber of hemocytometer. The absolute number of cells is shown in (A) bone marrow (BM) and (B) spleen (SP) of mice. Wild-type mice are shown in white bars and cathepsin K knockout (CatK KO) in black bars. Number of hematopoietic stem cells (Lin$^-$ c-Kit$^+$ Sca-1$^+$, LKS) as determined by flow cytometry are shown. Lineage markers included are B220, CD8, CD4, CD3, CD11b, F4/80, Gr-1, DX5, and Ter119. The percentages of HSCs among live cells are shown in bone marrow (C) and spleen (E). The total number of HSCs is shown as absolute numbers in (D) bone marrow and (F) spleen of wild-type and CatK KO mice. Data shown are a representative of n = 4 (WT) and n = 4 (CatK KO). *$P < 0.05$, ***$P < 0.0005$ as determined by using Student's *t*-test. $P < 0.05$ was considered statistically significant.

Next, we determined whether CatK deficiency influenced hematopoiesis in the bone marrow. Using FACS analysis, HSCs were identified as Lin$^-$ c-Kit$^+$ Sca-1$^+$ cells. Data were analyzed

in two ways. (1) We measured the percentage change to understand whether changes in a population are specific to that population. (2) We measured the absolute number of cells in a population to understand whether any observed changes in the number of cells were due to an overall change in cellularity.

The percentage of HSCs among total live cells in the bone marrow of CatK$^{-/-}$ mice was not significantly different from that in WT mice (Fig. 1C). However, the absolute number of HSCs was decreased by twofold in the bone marrow of CatK$^{-/-}$ mice compared to WT mice (Fig. 1D). Previous reports demonstrated that osteopetrotic mice have enhanced extramedullary hematopoiesis [Lowell et al., 1996; Tagaya et al., 2000]. Consistent with these reports, we found that both the percentage and absolute number of HSCs were increased by threefold in the spleen of CatK$^{-/-}$ mice compared to WT mice (Fig. 1E,F).

LOSS OF CATHEPSIN K ALTERS THE MONOCYTE SUBPOPULATIONS IN THE BONE MARROW

HSCs give rise to all lineages of blood cells including myeloid lineage cells, which, in turn, give rise to OCs [Arai et al., 1999]. We next examined the composition of myeloid populations to understand the effect that the absence of CatK in mice had on these cells. Myeloid cells were defined as negative for both the B cell marker B220 and the T cell marker CD3, and positive for the myeloid marker CD11b. These were further analyzed for the expression of markers of subsets of monocyte populations, LY6C and LY6G. Similar to the number of HSCs, the percentages of monocyte subsets (LY6C high and low, LY6G high and low) were similar in the bone marrow of CatK$^{-/-}$ mice when compared to WT mice (Fig. 2A–D). However, the absolute numbers of these monocyte populations were lower in the bone marrow of CatK$^{-/-}$ mice as compared to WT mice suggesting that the decrease in bone marrow cellularity in CatK$^{-/-}$ mice is associated

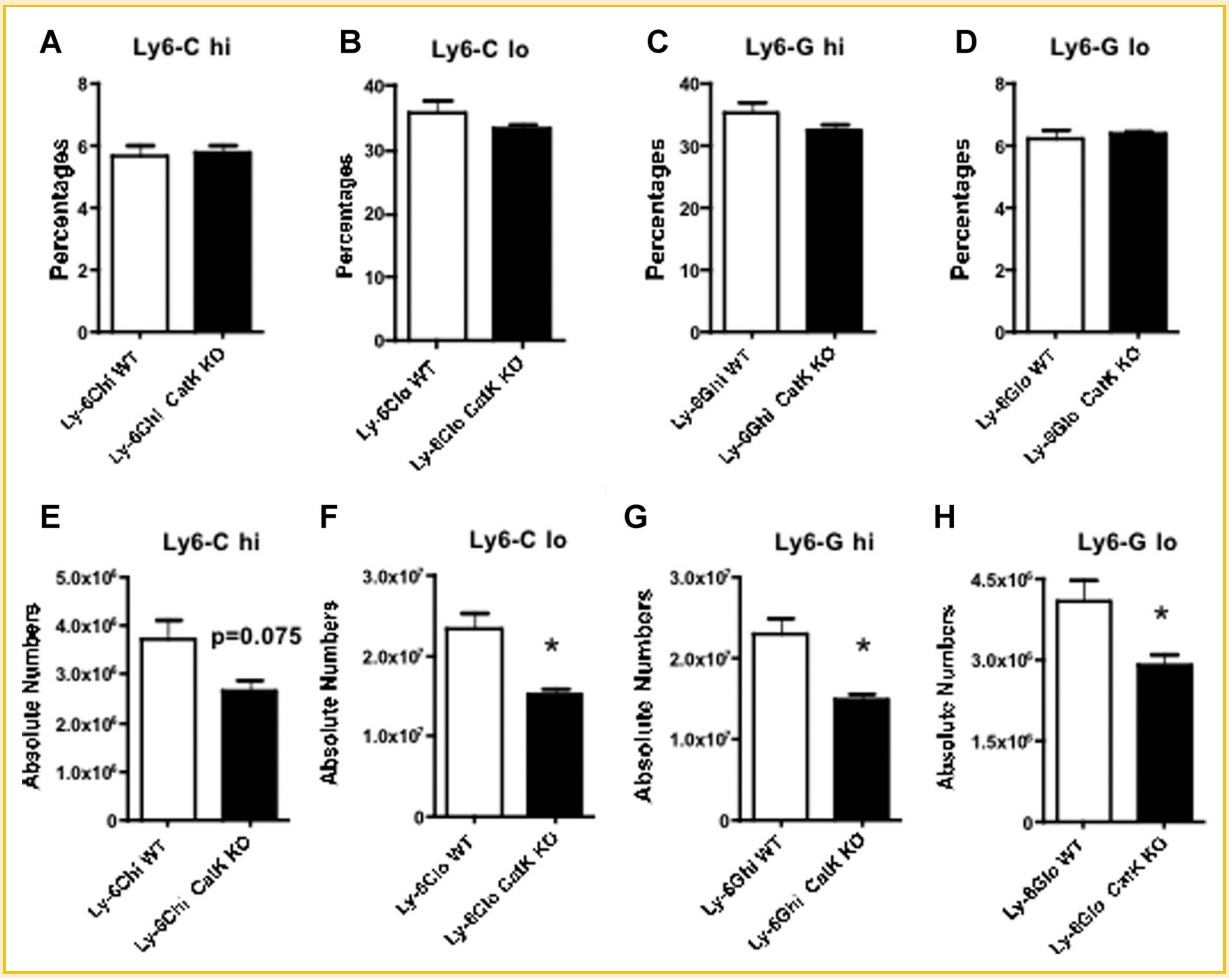

Fig. 2. Number of monocyte subpopulations in the bone marrow of mice lacking cathepsin K. Myeloid lineage cells (B220$^-$ CD3$^-$ CD11b$^+$) in the bone marrow were gated for the expression of monocyte markers Ly6C and LY6G and the number of cells expressing high levels of Ly6C (Ly6-C hi) and Ly6G (Ly6-G hi) or low levels of Ly6C (Ly6-C lo) and Ly6G (Ly6-G lo) were determined. Wild-type mice are shown in white bars and cathepsin K knockout (CatK KO) in black bars. The percentage of monocyte subpopulations among live cells in the bone marrow are shown for (A) Ly6-C hi cells, (B) Ly6-C lo, (C) Ly6-G hi, (D) Ly6-G lo and the total number of monocyte subpopulations in the bone marrow are shown as absolute numbers for (E) Ly6-C hi cells (F) Ly6-C lo (G) Ly6-G hi (H) Ly6-G lo. Data shown are a representative of n = 4 (WT) and n = 4 (CatK KO). *$P < 0.05$ as determined by using Student's *t*-test was considered statistically significant.

with decreases in the absolute number of monocyte subpopulations in the bone marrow (Fig. 2E–H).

ABSENCE OF CATHEPSIN K IN MICE DECREASES THE NUMBER OF MACROPHAGES AND DENDRITIC CELLS IN THE BONE MARROW

In order to evaluate whether the decrease in monocyte subpopulations is unique to these subsets or whether loss of CatK influences multiple myeloid lineage cells, we also analyzed the number of macrophages expressing the antigen F4/80 and dendritic cells expressing the antigen CD11c. Both the percentages and absolute number of macrophages (Fig. 3A,C) and dendritic cells (Fig. 3B,D) were decreased in the bone marrow of $CatK^{-/-}$ mice. The decrease in the number of macrophages and dendritic cells in CatK-deficient bone marrow was not just due to a decrease in overall bone marrow cellularity as the percentage of these cells relative to all bone marrow cells was also significantly decreased.

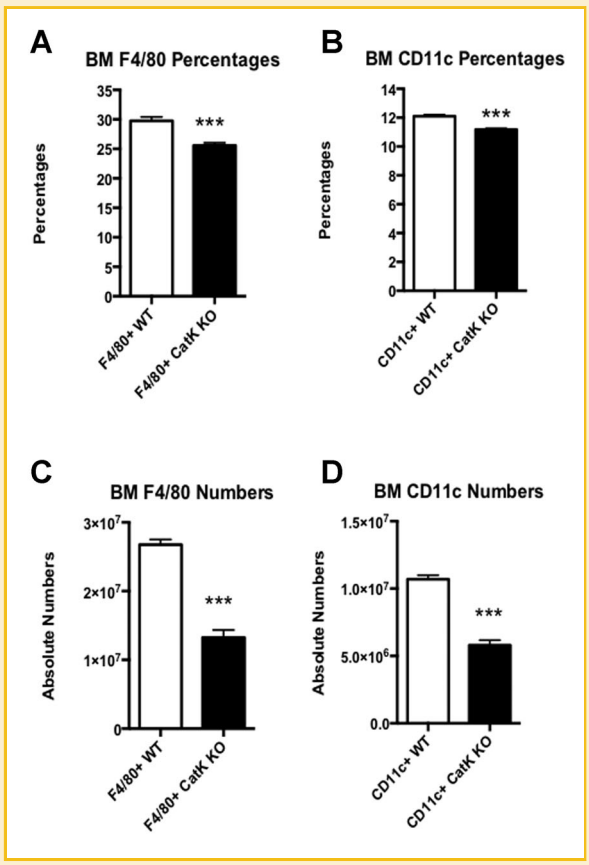

Fig. 3. Number of macrophages and dendritic cells in the bone marrow of mice lacking cathepsin K. Macrophages were identified by FACS analysis as $B220^-$ $CD3^-$ $CD11b^+$ $F4/80^+$ cells and dendritic cells as $CD11c^+$ cells in the bone marrow (BM). Wild-type mice are shown in white bars and cathepsin K knockout (CatK KO) in black bars. Percentage among live cells in bone marrow as shown for (A) macrophages (B) dendritic cells (C) and the total number of cells are shown as absolute numbers for (D) macrophages and (E) dendritic cells. Data shown are a representative of n = 4 (WT) and n = 4 (CatK KO). ***$P < 0.0005$ as determined by using Student's *t*-test. $P < 0.05$ was considered statistically significant.

ABSENCE OF CATHEPSIN K IN MICE DOES NOT ALTER THE EARLY OSTEOCLAST PRECURSORS IN THE BONE MARROW BUT DOES AFFECT MORE MATURE CELLS

CatK mice have an increased number of defective OCs [Kiviranta et al., 2005; Soung do et al., 2013]. In addition, our data demonstrate that the bone marrow of CatK-deficient mice have a decreased number of HSCs and myeloid cells. To determine whether CatK-deficiency also altered the number of OC precursors, we analyzed populations of OCPs in the bone marrow, which we previously defined based on their capacity to differentiate in vitro into mature OCs with high efficiency after treatment with M-CSF and RANKL [Jacquin et al., 2006; Jacome-Galarza et al., 2011, 2013].

Our published paradigm defines early OCPs as cells which are negative for both the B cell marker B220 and the T cell marker CD3, express low levels of CD11b, high levels of the M-CSF receptor, CD115, and high (population IV), intermediate (population V) or low levels (population VI) of the early hematopoietic progenitor cell marker CD117 (c-kit) [Jacquin et al., 2006]. We found no differences in either the percentage or the absolute number of the three subsets of early bone marrow OCPs (populations IV, V, or VI) between CatK deficient and WT mice (Fig. 4A,B).

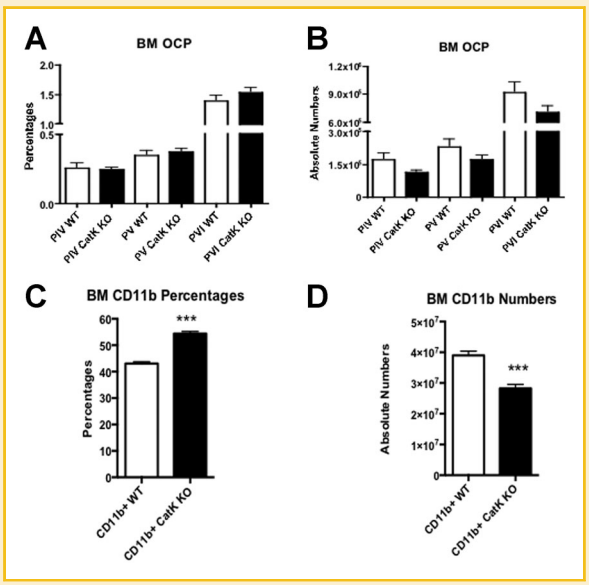

Fig. 4. Number of early and late precursors of osteoclasts in the bone marrow of mice lacking cathepsin K. Early osteoclast precursors (OCP) were identified by FACS analysis as $B220^-$ $CD3^-$ $CD11b^{lo}$ cells expressing CD115 with high levels of CD117 ($CD117^+$: Population IV, PIV), low levels of CD117 ($CD117^{lo}$: Population V, PV) or no CD117 ($CD117^-$: population VI, PVI). A: Percentage of different subsets among live cells in the bone marrow (BM) is shown. B: The total number of osteoclast precursor subsets in the bone marrow is shown as absolute numbers. Late osteoclast precursors were identified as $B220^-CD3^-$ cells committed to myeloid lineage ($CD11b^+$) and (C) the percentage among live cells or (D) the total number represented as absolute numbers in the bone marrow are shown. Wild-type mice are shown in white bars and cathepsin K knockout (CatK KO) in black bars. Data shown are a representative of n = 4 (WT) and n = 4 (CatK KO). ***$P < 0.0005$ as determined by using Student's *t*-test. $P < 0.05$ was considered statistically significant.

In order to understand whether the commitment of myeloid lineage cells is skewed toward more committed late OCPs, we analyzed the number of cells expressing high levels of CD11b in bone marrow. We previously found that cells in populations IV, V, and VI express high levels of CD11b later in their differentiation after exposure to M-CSF [Jacquin et al., 2006; Jacome-Galarza et al., 2011, 2013]. Similar to the decrease in total cellularity in the bone marrow, the absolute number of $CD11b^{hi}$ cells was decreased (Fig. 4D). However, the percentage of bone marrow cells expressing $CD11b^{hi}$ was significantly higher in $CatK^{-/-}$ mice (Fig. 4C). These results suggest that increased number of OCs in $CatK^{-/-}$ mice may result from an increased commitment toward late OCPs in bone marrow cells.

THE NUMBER OF MACROPHAGES BUT NOT DENDRITIC CELLS AND LATE OSTEOCLAST PRECURSORS ARE INCREASED IN THE SPLEEN OF MICE LACKING CATHEPSIN K

Since hematopoiesis has been demonstrated to increase in extramedullary tissue in mice with an osteopetrotic phenotype [Lowell et al., 1996; Tagaya et al., 2000], and we found the absolute number and percentages of HSCs were higher in the spleens of $CatK^{-/-}$ mice, we determined the number of myeloid cells in $CatK^{-/-}$ mice spleens. The goal was to understand whether the changes observed in myeloid composition of bone marrow are tissue specific.

In contrast to bone marrow, which demonstrated a decrease in both the percentage and absolute number of F4/80 positive cells, the percentage and absolute number of F4/80 positive macrophages (Fig. 5B,E) in the spleen was significantly increased in $CatK^{-/-}$ mice with no differences in late OCPs ($CD11b^{hi}$ cells, Fig. 5A,D) or dendritic cells (CD11c positive cells, Fig. 5C,F) when compared to WT mice cells. These results suggest that the loss of CatK selectively alters the myeloid compartment in the bone marrow, which is in proximity to defective bone remodeling environment. This increase in proximity also results in increased OC formation while at the same time selectively increasing macrophage development in the spleen.

We previously identified $LY6C^{hi}$ $CD115^{+}$ cells in the spleen and blood as having high osteoclastogenic potential [Jacome-Galarza et al., 2011]. We found that in $CatK^{-/-}$ mice the percentages but not the absolute number of these cells in the spleen was decreased compared to WT mice (Fig. 6A,B). Since the percentages of $LY6C^{hi}$ populations were not altered in the bone marrow but was decreased in the spleen, we analyzed their levels in the peripheral blood. We found that the percentages of $Ly6C^{hi}$ cells were similar in the peripheral blood of $CatK^{-/-}$ mice compared to the peripheral blood

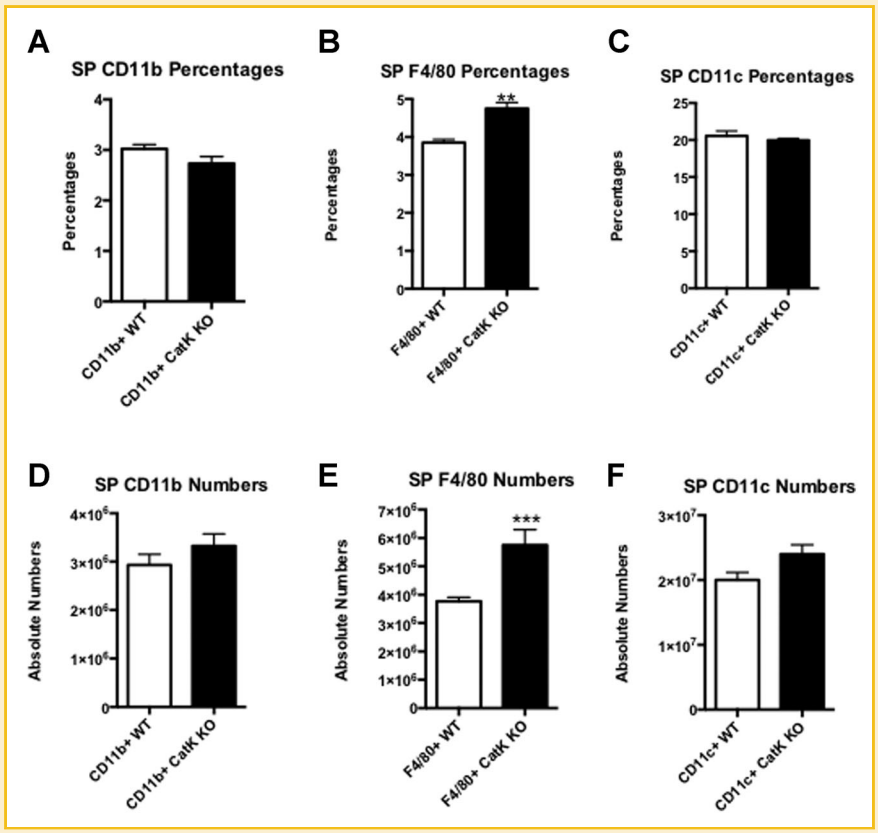

Fig. 5. Number of myeloid cells, macrophages, and dendritic cells in the spleen of mice lacking cathepsin K. Myeloid cells were identified by FACS analysis as $B220^{-}$ $CD3^{-}$ $CD11b^{+}$ cells, macrophages as $F4/80^{+}$ and dendritic cells as $CD11c^{+}$ cells in the spleen (SP). Wild-type mice are shown in white bars and cathepsin K knockout (CatK KO) in black bars. Percentage among live cells in spleen as shown for (A) myeloid cells (B) macrophages (C) dendritic cells and the total number of cells are shown as absolute numbers for (D) myeloid cells (E) macrophages and (F) dendritic cells. Data shown are a representative of n = 4 (WT) and n = 4 (CatK KO). $^{**}P < 0.005$, $^{***}P < 0.0005$ as determined by using Student's *t*-test. $P < 0.05$ was considered statistically significant.

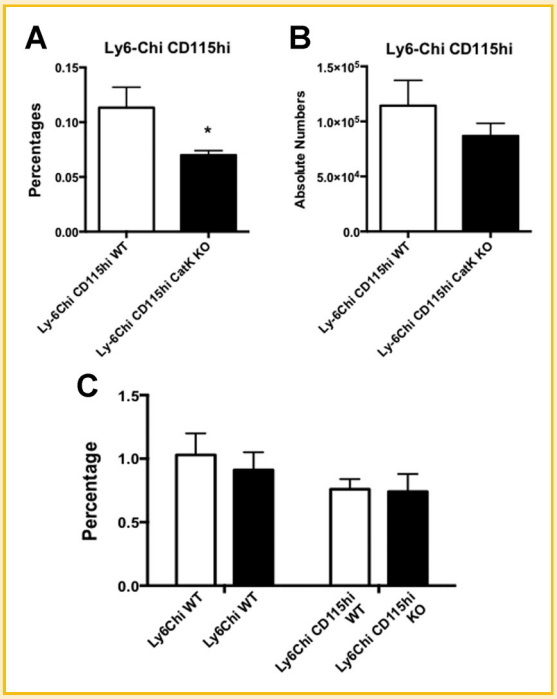

Fig. 6. Number of osteoclast precursors in the spleen and peripheral blood of mice lacking cathepsin K. Osteoclast precursors (OCPs) were identified by FACS analysis as $B220^{-}$ $CD3^{-}$ $NK1.1^{-}$ $CD11b^{+}$ cells which express high (hi) levels of CD115 and LY6-C. A: Percentage of OCPs among live cells in spleen are shown. Wild-type mice are shown in white bars and cathepsin K knockout (CatK KO) in black bars. B: The total number of OCPs is shown as absolute numbers in spleen. C: Percentage of OCPs among live cells in the peripheral blood is shown. Data shown are a representative of n = 4 (WT) and n = 4 (CatK KO). *$P < 0.05$ as determined by using Student's *t*-test was considered statistically significant.

of WT cells (Fig. 6C). Similarly, the percentages of $LY6C^{hi}$ $CD115^{hi}$ OCPs were similar in the peripheral blood of $CatK^{-/-}$ and WT mice (Fig. 6C).

$CatK^{-/-}$ OC PRECURSOR CELLS HAVE A DECREASED CAPACITY TO MIGRATE AND ENGRAFT IN BONE AT THE SITE OF A REPAIRING FRACTURE CALLUS

In order to understand whether the increased commitment of monocyte precursors to the OC lineage in $CatK^{-/-}$ mice is due the osteopetrotic environment, $CatK^{cre/cre}$ or $CatK^{cre/+}$ OCPs were injected into WT mice, which also underwent fracture at the mid-diaphysis of their femurs. In these experiments, the injected OCPs contain cre recombinase that replaced the CatK gene and express a red fluorescent label of TD-tomato after recombination.

In these studies, OCs in the fracture callus, expressing red fluorescence, are derived from OCPs that are injected intravenously at the time of fracture and 10 days later. Animals are then sacrificed at day 18 post-fracture, when the repair callus is maximally reorganizing from trabecular to cortical bone. We verified that bone marrow cells from $CatK^{cre/+}$ TD-tomato mice express little fluorescence when cultured with M-CSF alone. However, when these cells were cultured with M-CSF + RANKL for 5 days, both mono- and multinucleated fluorescent TRAP-positive cells develop (data not shown). In WT mice, which were injected intravenously with $CatK^{cre/+}$ TD-tomato bone marrow, numerous nests of red fluorescent OCs were detected in the fracture callus at day 18 post-fracture. In contrast, when $CatK^{cre/cre}$ TD-tomato OCPs were injected into WT mice, significantly fewer red fluorescent OCs were detected in the repair callus at day 18 post-fracture (Fig. 7A,B).

When the sections were subjected to TRAP staining, a similar number of TRAP+ OCs were detected in the repair callus of mice that received either type of cells (Fig. 7C,D), since both endogenous WT OCPs and injected OCPs have been demonstrated to be equally capable of differentiating into TRAP-positive OCs in vitro. These results suggest that after intravenous injection OC precursor cells, which are deficient in CatK, have a defect in their ability to migrate to or engraft at the active remodeling site of a repair fracture callus.

DISCUSSION

The effect of CatK on osteoclastogenesis has been documented both in knock-out mice and in studies using CatK inhibitor treatments, where the genetic or pharmacological inhibition of CatK resulted in increased formation of OCs with defective resorption capacity [Kiviranta et al., 2005; Chen et al., 2007; Masarachia et al., 2012; Soung do et al., 2013]. It has been suggested that the increases in RANKL to OPG ratio in the local bone marrow environment in $CatK^{-/-}$ mice may favor the enhanced osteoclastogenesis [Kiviranta et al., 2005]. However, the mechanism by which CatK inhibition increases the number of mature OCs is not clearly linked to enhanced commitment of early or late hematopoeitic progenitors to the OC lineage. Hence, we utilized FACS analysis to identify the hematopoietic and OC precursor population characteristics tracing the origin of the increased number of OCs from the HSCs to early and more committed OC precursors in WT mice and $CatK^{-/-}$ mice.

FACS analysis and in vivo OCP tracing studies reveal that the absence of CatK enhanced the commitment of monocyte precursors toward the more mature ($CD11b^{+}$) OC lineage only in the bone marrow. We also found that the number of HSCs decreased in the bone marrow and was higher in the spleen of $CatK^{-/-}$ mice compared to WT mice, which is consistent with the known decrease in the volume of the bone marrow space in osteopetrotic animals and the consequent development of extramedullary hematopoiesis [Lowell et al., 1996; Tagaya et al., 2000].

We further found that the pattern of myeloid cells was different in the spleen compared to bone marrow. In the spleen, only mature $F4/80^{+}$ macrophages were increased in percentage and absolute number in $CatK^{-/-}$ mice, whereas late OC precursors and dendritic cells were similar to that of WT mice. In the blood, where we could only measure the percentage of each population, there were no differences in our previously characterized OC precursor populations [Jacome-Galarza et al., 2011].

We have demonstrated that treatment with a cathepsin K inhibitor increased the absolute number of OCs within repairing fracture calluses [Soung do et al., 2013]. Since the bone remodeling microenvironment is intricately dependent on the recruitment and

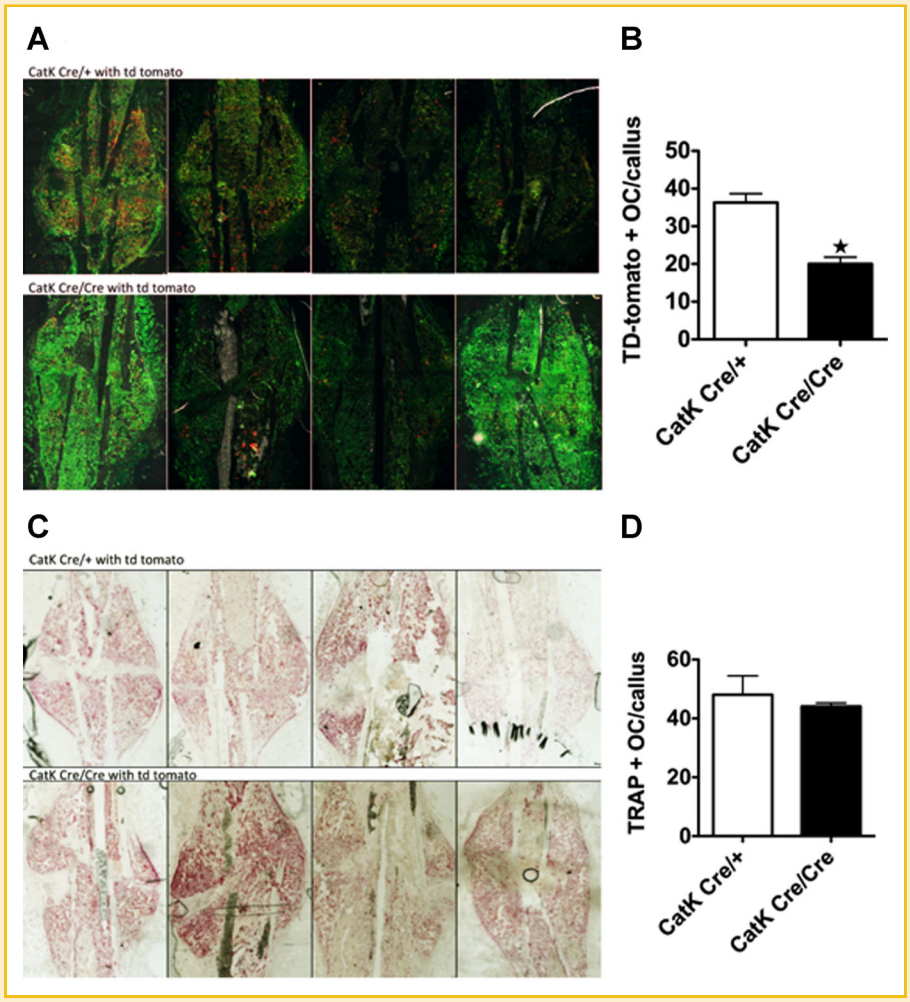

Fig. 7. In vivo tracking of osteoclast precursors from peripheral blood to bone in the repairing fracture callus. A: Red fluorescence from TD-tomato expression in osteoclasts in repairing fracture callus are shown in mice which received $CaK^{Cre/+}$ OCPs (top panel) or $CaK^{Cre/Cre}$ OCPs in the bottom panel. B: The number of red fluorescent cells was determined by cell counting performed using image J pro. Cells were counted on sections from four individual mice and the average number of TD-tomato positive red fluorescent cells per callus is shown. CatK Cre/+, OCPs expressing CatK were injected into wild-type mice, CatK Cre/Cre, OCPs lacking CatK were injected into wild-type mice. (C) TRAP staining of the fracture callus of wild-type mice injected with CatK Cre/+ OCPs (top panel) or CatK Cre/Cre OCPs (bottom panel). D: TRAP+ cells were counted and the number of cells per callus is presented. Data shown are a representative of n = 4 (WT) and n = 4 (CatK KO). $^*P < 0.05$, as determined by using Student's *t*-test. $P < 0.05$ was considered statistically significant.

activity of OCs, osteoblasts and canopy lining cells [Schindeler et al., 2008; Sims and Gooi, 2008], introduction of $CatK^{-/-}$ OCPs into a WT remodeling environment in a fracture callus might address the question of whether signals from the local bone remodeling environment are responsible for the increased number of OCs. Adoptive transfer of red fluorescent OCPs expressing or not expressing CatK into WT mice subjected to fracture, resulted in formation of numerous red fluorescent OCs in the fracture callus when the transferred cells were heterozygous for CatK expression ($CatK^{cre/+}$). In contrast, the number of fluorescent OCs in the repairing fracture callus was much lower when CatK-deficient OCPs were injected into the WT mice. These results demonstrate a defect in the ability of CatK-deficient OCPs ($CatK^{cre/cre}$) to either migrate or engraft into active bone remodeling units in this model. Hence, it is unlikely that enhanced recruitment of circulating OCPs is involved in the increased OC number that is seen in the bones of $CatK^{-/-}$ mice. Rather, our data suggest that $CD11b^{+}$ OCPs in the bone marrow, which are locally increased in $CatK^{-/-}$ mice, are involved in the increased osteoclastogenesis that is seen in these mice. This finding is consistent with the local increase in the ratio of RANKL to OPG reported in $CatK^{-/-}$ mice [Kiviranta et al., 2005]. It may also be a consequence of the enhanced bone formation that occurs in the bone marrow microenvironment of $CatK^{-/-}$ mice as a result of the enhance production of sphingosine-1-phosphate by cathepsin-K-deficient OCs [Lotinun et al., 2013]. In our studies the $CatK^{-/-}$ cells that migrated and engrafted at a fracture repair site represented only a very small fraction of the TRAP-positive cells in the bone, which were otherwise overwhelmingly $CatK^{+/+}$. Hence, it was anticipated

there we would find no differences in total OC number in the repair callus of mice in which either CatK$^{-/-}$ or CatK$^{+/-}$ cells were injected into fractured CatK$^{+/+}$ mice.

Taken together, our findings suggest that the increased CD11b^{+} cells in bone marrow and OC number on bone, which occurs in CatK$^{-/-}$ mice, are influenced by increases in local cytokine production that drive myeloid bone marrow precursor toward the OC lineage.

REFERENCES

Arai F, Miyamoto T, Ohneda O, Inada T, Sudo T, Brasel K, Miyata T, Anderson DM, Suda T. 1999. Commitment and differentiation of osteoclast precursor cells by the sequential expression of c-Fms and receptor activator of nuclear factor kappaB (RANK) receptors. J Exp Med 190:1741–1754.

Calvi LM, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC, Martin RP, Schipani E, Divieti P, Bringhurst FR, Milner LA, Kronenberg HM, Scadden DT. 2003. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature 425:841–846.

Chen W, S. Yang Y Abe, M. Li Y Wang, J. Shao E Li. 2007. Novel pycnodysostosis mouse model uncovers cathepsin K function as a potential regulator of osteoclast apoptosis and senescence. Hum Mol Genet 16:410–423.

Jacome-Galarza CE, Lee SK, Lorenzo JA, Aguila HL. 2013. Identification, characterization, and isolation of a common progenitor for osteoclasts, macrophages, and dendritic cells from murine bone marrow and periphery. J Bone Miner Res 28:1203–1213.

Jacome-Galarza CE, Lee SK, Lorenzo JA, Aguila HL. 2011. Parathyroid hormone regulates the distribution and osteoclastogenic potential of hematopoietic progenitors in the bone marrow. J Bone Miner Res 26:1207–1216.

Jacquin C, Gran DE, Lee SK, Lorenzo JA, Aguila HL. 2006. Identification of multiple osteoclast precursor populations in murine bone marrow. J Bone Miner Res 21:67–77.

Kaback LA, Soung do Y, Naik A, Geneau G, Schwarz EM, Rosier RN, O'Keefe RJ, Drissi H. 2008a. Teriparatide (1-34 human PTH) regulation of osterix during fracture repair. J Cell Biochem 105:219–226.

Kaback LA, Soung do Y, Naik A, Smith N, Schwarz EM, O'Keefe RJ, Drissi H. 2008b. Osterix/Sp7 regulates mesenchymal stem cell mediated endochondral ossification. J Cell Physiol 214:173–182.

Kiviranta R, Morko J, Alatalo SL, NicAmhlaoibh R, Risteli J, Laitala-Leinonen T, Vuorio E. 2005. Impaired bone resorption in cathepsin K-deficient mice is partially compensated for by enhanced osteoclastogenesis and increased expression of other proteases via an increased RANKL/OPG ratio. Bone 36:159–172.

Kollet O, Dar A, Shivtiel S, Kalinkovich A, Lapid K, Sztainberg Y, Tesio M, Samstein RM, Goichberg P, Spiegel A, Elson A, Lapidot T. 2006. Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells. Nat Med 12:657–664.

Kollet O, Dar Lapidot AT. 2007. The multiple roles of osteoclasts in host defense: Bone remodeling and hematopoietic stem cell mobilization. Annu Rev Immunol 25:51–69.

Li S, Q. Zhai D Zou, H. Meng Z Xie, C. Li Y Wang, J. Qi T Cheng, Qiu L. 2013. A pivotal role of bone remodeling in granulocyte colony stimulating factor induced hematopoietic stem/progenitor cells mobilization. J Cell Physiol 228:1002–1009.

Lotinun S, Kiviranta R, Matsubara T, Alzate JA, Neff L, Luth A, Koskivirta I, Kleuser B, Vacher J, Vuorio E, Horne WC, Baron R. 2013. Osteoclast-specific cathepsin K deletion stimulates S1P-dependent bone formation. J Clin Invest 123:666–681.

Lowell CA, Niwa M, Soriano P, Varmus HE. 1996. Deficiency of the Hck and Src tyrosine kinases results in extreme levels of extramedullary hematopoiesis. Blood 87:1780–1792.

Lymperi S, Ersek A, Ferraro F, Dazzi F, Horwood NJ. 2011. Inhibition of osteoclast function reduces hematopoietic stem cell numbers in vivo. Blood 117:1540–1549.

Mansour A, Abou-Ezzi G, Sitnicka E, Jacobsen SE, Wakkach A, Blin-Wakkach C. 2012. Osteoclasts promote the formation of hematopoietic stem cell niches in the bone marrow. J Exp Med 209:537–549.

Masarachia PJ, Pennypacker BL, Pickarski M, Scott KR, Wesolowski GA, Smith SY, Samadfam R, Goetzmann JE, Scott BB, Kimmel DB, Duong le T. 2012. Odanacatib reduces bone turnover and increases bone mass in the lumbar spine of skeletally mature ovariectomized rhesus monkeys. J Bone Miner Res 27:509–523.

Naik AA, Xie C, Zuscik MJ, Kingsley P, Schwarz EM, Awad H, Guldberg R, Drissi H, Puzas JE, Boyce B, Zhang X, O'Keefe RJ. 2009. Reduced COX-2 expression in aged mice is associated with impaired fracture healing. J Bone Miner Res 24:251–264.

Nakamura T, Imai Y, Matsumoto T, Sato S, Takeuchi K, Igarashi K, Harada Y, Azuma Y, Krust A, Yamamoto Y, Nishina H, Takeda S, Takayanagi H, Metzger D, Kanno J, Takaoka K, Martin TJ, Chambon P, Kato S. 2007. Estrogen prevents bone loss via estrogen receptor alpha and induction of Fas ligand in osteoclasts. Cell 130:811–823.

Pennypacker B, Shea M, Liu Q, Masarachia P, Saftig P, Rodan S, Rodan G, Kimmel D. 2009. Bone density, strength, and formation in adult cathepsin K (−/−) mice. Bone 44:199–207.

Saftig P, Hunziker E, Wehmeyer O, Jones S, Boyde A, Rommerskirch W, Moritz JD, Schu P, von Figura K. 1998. Impaired osteoclastic bone resorption leads to osteopetrosis in cathepsin-K-deficient mice. Proc Natl Acad Sci USA 95:13453–13458.

Schindeler A, McDonald MM, Bokko P, Little DG. 2008. Bone remodeling during fracture repair: The cellular picture. Semin Cell Dev Biol 19:459–466.

Shivtiel S, Kollet O, Lapid K, Schajnovitz A, Goichberg P, Kalinkovich A, Shezen E, Tesio M, Netzer N, Petit I, Sharir A, Lapidot T. 2008. CD45 regulates retention, motility, and numbers of hematopoietic progenitors, and affects osteoclast remodeling of metaphyseal trabecules. J Exp Med 205:2381–2395.

Sims NA, Gooi JH. 2008. Bone remodeling: Multiple cellular interactions required for coupling of bone formation and resorption. Semin Cell Dev Biol 19:444–451.

Soung do Y, Talebian L, Matheny CJ, Guzzo R, Speck ME, Lieberman JR, Speck NA, Drissi H. 2012. Runx1 dose-dependently regulates endochondral ossification during skeletal development and fracture healing. J Bone Miner Res 27:1585–1597.

Soung do Y, Gentile MA, Duong le T, Drissi H. 2013. Effects of pharmacological inhibition of cathepsin K on fracture repair in mice. Bone 55:248–255.

Tagaya H, Kunisada T, Yamazaki H, Yamane T, Tokuhisa T, Wagner EF, Sudo T, Shultz LD, Hayashi SI. 2000. Intramedullary and extramedullary B lymphopoiesis in osteopetrotic mice. Blood 95:3363–3370.

Teitelbaum SL. 2000. Bone resorption by osteoclasts. Science 289: 1504–1508.