Osteoclasts are not crucial for hematopoietic stem cell maintenance in adult mice

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ABSTRACT

The osteoclast is vital for establishment of normal hematopoiesis in the developing animal. However, its role for maintenance of hematopoiesis in adulthood is more controversial. To shed more light on this process, we transplanted hematopoietic stem cells from two osteopetrotic mouse models, with lack of osteoclasts or defective osteoclast function, to normal adult mice and examined the bone phenotype and hematopoiesis in the recipients. B6SJL mice were lethally irradiated and subsequently transplanted with oc/oc, Receptor Activator of Nuclear Factor Kappa B knockout or control fetal liver cells. Osteoclasts derived from the recipient animals were tested *in vitro* for osteoclastogenesis and resorptive function. Bone remodeling changes were assessed using biomarkers of bone turnover and micro-CT. Hematopoiesis was assessed by flow cytometry and colony formation, and hematopoietic stem cell function by secondary competitive transplantations and cell cycle analysis. After transplantation, a donor chimerism of 97-98% was obtained, and by 15 weeks mild osteopetrosis had developed in recipients of cells from osteopetrotic mice. There were no alterations in the number of bone marrow cells. Colony formation was slightly reduced in Receptor Activator of Nuclear Factor Kappa B knock-out recipients but unchanged in oc/oc recipients. Phenotypically, stem cells were marginally reduced in recipients of cells from osteopetrotic mice, but no significant difference was seen in cell cycle status and in competitive secondary transplantations all three groups performed equally well. Our results indicate that osteoclast function is not crucial for hematopoietic stem cell maintenance in adult mice.

Introduction

The osteoclast responsible for the resorption of bone and the osteoblast ensuring formation of new bone are two unique cell types that continuously repair and maintain the human skeleton through a tightly co-ordinated process known as bone remodeling. During ontogeny, both osteoclasts and osteoblasts are essential for the formation of the specialized microenvironmental niche where the blood-forming hematopoietic stem cells reside, the hematopoietic niche.[1,2] The hematopoietic stem cells (HSCs) interaction with their microenvironment is critical when maintaining normal hematopoiesis and their specific fate is determined through complex, bidirectional interactions with various cell types and stromal cell components.[3-5] In the adult bone marrow (BM), different stromal cells regulate HSCs. Osteoblasts maintain the HSCs in an undifferentiated, quiescent state by providing inhibitory signals like Jagged and Angiopoietin 1, but also by expressing N-cadherin and VCAM that interact with integrins expressed on HSCs, attaching them to the niche.[6-11] Vascular stromal cells, e.g. sinusoidal endothelial cells,[12] fibroblast-like reticular cells and Nestin+ mesenchymal stem cells that express high levels of SDF-1/CXCL12 also play key roles in HSC maintenance.[13-17]

Lately, several reports have highlighted the importance of the osteoclast in regulation of the hematopoietic niche, but its precise role for this process under various conditions still remains controversial. It has been shown that osteoclast-mediated resorption promotes mobilization of HSCs and progenitors from the niche to the circulation by cathepsin K-mediated cleavage of CXCL12.[18] In contrast to this, osteoclast inhibition was also shown to increase mobilization.[19,20] In addition, it has been demonstrated that mice lacking calcium-sensing receptors have reduced numbers of HSCs in the BM, indicating that the calcium released as a consequence of bone resorption is important for the correct localization of HSCs and that this is specified by calcium-sensing receptors.[21,22] Furthermore, when normal mice were treated with the bisphosphonate alendronate (that inhibits and induces apoptosis in osteoclasts), a slight reduction of HSCs in the BM was observed.[23]

In the present study, aiming to explore the role of the osteoclast for maintenance of adult hematopoiesis, two osteopetrotic mouse models were used: the oc/oc and RANK KO. Oc/oc mice with a mutation in the *Tcirg1* gene lack osteoclastic V-ATPase activity and their resorptive function has been completely abolished, but they do have a large number of osteoclasts and a severe osteopetrotic phenotype with a short life expectancy of 3-4 weeks.[24] In contrast, the RANK KO mouse is defective in osteoclast differentiation and is, therefore, devoid of osteoclasts. Both models suffer from osteopetrosis, but the phenotype is less severe and the life expectancy is longer in the RANK KO than in the oc/oc mouse.[25] By irradiating wild-type mice, and subsequently transplanting fetal liver cells from either oc/oc or RANK KO mice, we generated adult mice with osteopetrosis suitable for

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studying the role of osteoclasts for maintenance of hematopoiesis in this setting.

Methods

Mice

Breeding pairs of oc/+ mice (CD45.2)[26] and B6SJL (CD45.1) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). RANK$^{+/-}$ mice (CD45.2) were obtained from Amgen (Seattle, WA, USA).[25] All experiments were performed according to protocols approved by the local animal ethics committee (number 333-11).

Genotyping of mice

Mice were genotyped by PCR of tail tips, as described previously.[27]

Fetal liver cell harvesting

Fetal liver cells were collected as described previously.[28]

Primary transplantations

Three-month old B6SJL (CD45.1) recipients were transplanted with 2 million freshly thawed FL cells (CD45.2) administered by tail vein injection after lethal irradiation (950 cGy). Post transplant mice were treated with ciprofloxacin.

Secondary transplantations

A total of 2 x 10^5 BM cells (CD45.2) were harvested from primary recipients and transplanted into secondary recipients (CD45.1) in a competitive setting with 3 x 10^5 wild-type BM cells (CD45.1/2).

Flow cytometry analysis of peripheral blood, bone marrow and fetal liver cells

For engraftment, cells were stained with Ly5.1-PeCy5 and Ly5.2-FITC. For lineage analysis and stem cell analysis, cells were stained with a cocktail of antibodies directed against lineage markers, Sca1, c-Kit and SLAM-markers, as described in the *Online Supplementary Methods*.

Cell cycle analysis

For the cell cycle analysis of BM, antibodies were directed against lineage-positive cells (CD4, CD5, GR1, Lyt2, Ter119, CD11b and CD45R/b220)-PeCy5, c-kit-APC780, Sca1-APC, CD48-FITC, CD150-PeCy7, Ki67-PE and DAPI-Pacific Blue. For the analysis of FL cells, the lineage cocktail was (CD3, Ter119, B220, Gr1)-PECy5 (Becton Dickinson) (*Online Supplementary Methods*).

Colony-forming unit cell (CFU-C) assay

Freshly isolated BM cells (20,000 cells) were plated in duplicate in 35 mm dishes in 1.5 mL of Complete Methocult Media (Stem Cell Technologies). Colonies were counted on Day 12.

Micro-computed tomography

The cortical and trabecular femoral bone architecture was evaluated using a high-resolution μCT scanner (μCT 35; Scanco Medical AG, Brüttisellen, Switzerland) (*Online Supplementary Methods*). Cortical bone volume fraction BV/TV and cortical thickness Ct.Th* were calculated using the software provided with the scanner (IPL, version 5.15). The asterisk denotes that the cortical thickness was computed with the direct method, i.e. without model assumptions.[29]

Splenocyte osteoclast differentiation and resorption

Splenocytes were isolated as previously described[30] and were differentiated into mature osteoclasts (*Online Supplementary Methods*). Resorption pits were visualized by staining with Mayer's hematoxylin. The resorbed area was measured using NewCAST software (Visiopharm, Hørsholm, Denmark) with an Olympus IX-70 microscope (Olympus, Center Valley, PA, USA).

TRAP staining of osteoclasts

Fixed osteoclasts in cell culture were stained using the Leukocyte TRAP staining kit (Sigma-Aldrich). Histological sections of bone were stained for TRAP, counterstained with Mayer's hematoxylin, and mounted with DPX.

Biomarkers

TRAP5b and CTX-I were measured in serum using mouse TRAP5b ELISA kit and RatLaps ELISA kit (Immunodiagnostics Systems), respectively, according to standard protocols.

Statistical analysis

Statistical analysis was performed either with unpaired two-sided Student's t-test, one-way ANOVA, Bonferroni or Mann-Whitney. *P<0.05; **P<0.01; ***P<0.001.

Results

Transplantation of oc/oc and RANK KO HSCs to wild-type mice leads to mild osteoclast-rich and osteoclast-poor osteopetrosis in recipients, respectively

Recipient wild-type mice were lethally irradiated and subsequently transplanted with FL cells from oc/oc, RANK KO or littermate control mice. The mice transplanted with FL cells from oc/oc and RANK KO will be referred to as oc/oc recipients (oc/oc-R) and RANK KO recipients (RANK KO-R), respectively, and mice that received WT (+/+) or heterozygous oc/+ or RANK +/- FL cells will be referred to as control recipients (Ctrl-R). When the frequency of LSK-SLAM HSCs and the cell cycle status in the LSK population were analyzed in the three donor cell populations, no difference was observed (*Online Supplementary Figure S1A and B*). Six weeks post transplantation the percentage of donor cells in PB reached 97-98% in all groups, and this level of engraftment was sustained at the time of termination, 15 weeks after transplantation, in both PB and BM.

Splenocytes harvested from oc/oc and control recipient mice developed into large multinucleated TRAP positive osteoclasts *in vitro*, whereas splenocytes from RANK KO recipients were TRAP negative and failed to fuse into osteoclasts (Figure 1A). Neither oc/oc osteoclasts nor RANK KO cells were able to form resorption pits when cultured on bovine bone slices, while resorption was seen in osteoclast cultures derived from splenocytes of control mice (Figure 1A and B).

In vivo assessment of osteoclast numbers confirmed the osteoclast-rich and osteoclast-poor phenotypes, as TRAP5b (a biomarker of osteoclasts) was markedly increased in the serum of the oc/oc recipient group and significantly reduced in the serum of the RANK KO recipient group (Table 1). With respect to bone resorption, the levels of the bone resorption marker CTX-1 were significantly reduced in the serum of mice from both experimen-

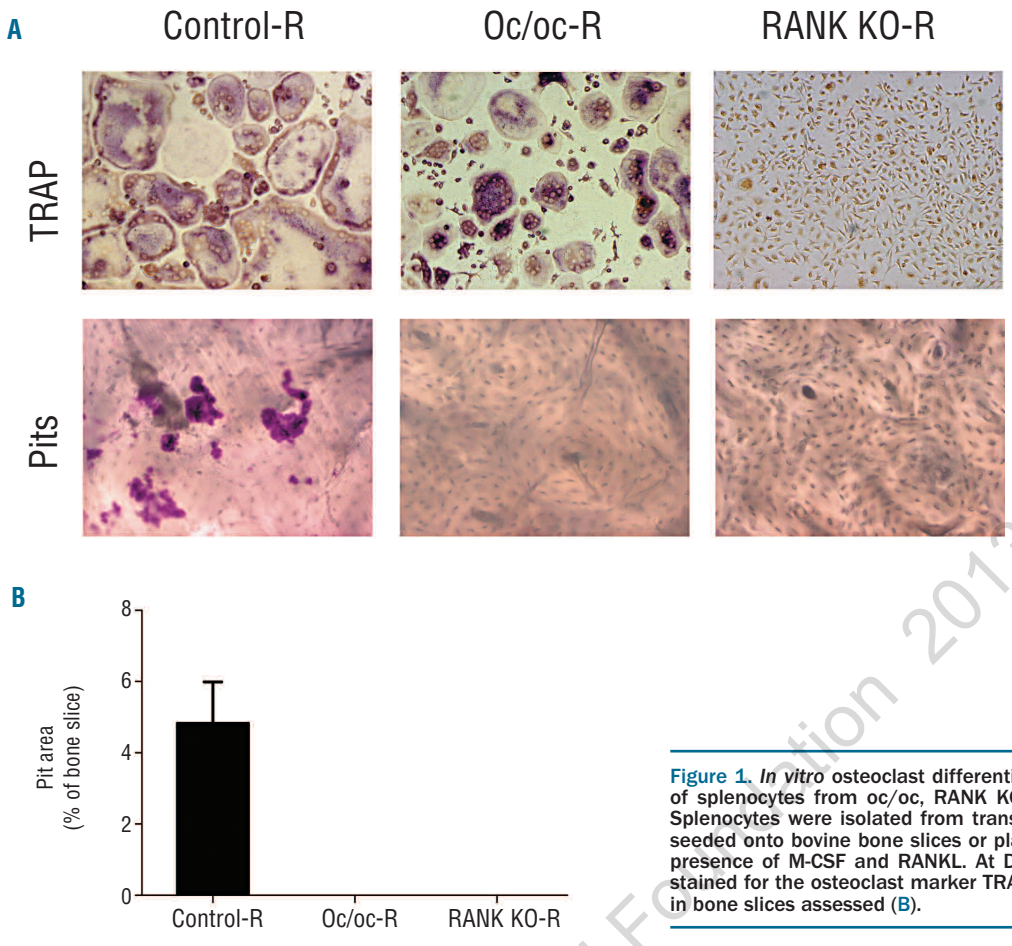

Figure 1. *In vitro* **osteoclast differentiation and resorption capacity of splenocytes from oc/oc, RANK KO and control recipient mice. Splenocytes were isolated from transplanted mice at termination, seeded onto bovine bone slices or plastic and differentiated in the presence of M-CSF and RANKL. At Day 10 cells were fixated and stained for the osteoclast marker TRAP (A), and the number of pits in bone slices assessed (B).**

Table 1. Bone volume and biomarker parameters of transplanted mice.

	Oc/oc recipients % of control	RANK KO recipients % of control
Cortical bone volume/tissue volume	108.4±2.3 (**)	107±1.6 (**)
Cortical thickness	110.2±4.1(**)	107.5±1.7 (*)
Serum TRAP5b	225.4±15.3 (***)	48.2±1.2 (***)
Serum CTX-I	83.9±2.1 (*)	83.6±3.1 (**)

Bone volume/tissue volume and cortical thickness were measured at the midshaft of the right femur as described in Methods. Biomarker measurements from weeks 6, 8, 12,14 and 15 were accumulated. For all parameters results are shown as % of control. Data were analyzed using a one-way ANOVA with a Bonferroni post test with P<0.05 considered significant and expressed as mean ± SEM. Oc/oc recipients (n=7), Rank KO recipients (n=12) and controls (n=21). Part of the data presented in this table will also be included in the manuscript in preparation by Thudium et al., *describing the osteopetrotic phenotypes in more detail.*

tal groups when compared to the control recipients (Table 1). To assess the bone phenotype of the two groups of transplanted mice, cortical BV/TV and cortical thickness were assessed at the femoral midshaft, and as shown there was a significant increase in both parameters in both the oc/oc and the RANK KO recipient groups when compared to the control recipients (Table 1). Furthermore, the magnitude of the increase was similar in the two groups, comparing well with a previous study using the oc/oc recipient mode.[28]

In summary, a mild osteopetrotic phenotype was observed in both the oc/oc recipient and the RANK KO recipient group; however, the former was osteoclast-rich and the latter was osteoclast-poor. Further analysis confirming the osteopetrotic phenotype in both these models has been performed (*CS Thudium et al., 2013, manuscript in preparation*).

BM cellularity is unaffected in both oc/oc and RANK KO recipients while progenitor cell number is slightly reduced in RANK KO recipients but unchanged in oc/oc recipients

When BM cellularity was evaluated by counting the total number of cells in one femur per recipient mouse sacrificed after 15 weeks, no difference was found between oc/oc, RANK KO and control recipients (Figure 2A). When the level of progenitor cells was assessed by CFU-formation, no difference was found between oc/oc and control recipients, whereas a slight reduction was seen in the RANK KO recipients as compared to control recipients (Figure 2B).

B220 positive B cells are reduced and myeloid cells increased in PB and BM of oc/oc recipients but not in RANK KO recipients

Prior to sacrifice of recipients at 15 weeks, a complete blood count was performed showing that the number of leukocytes and erythrocytes in both oc/oc and RANK KO recipients was comparable to that of control recipients (*data not shown*). However, when lineage analysis by FACS

of both PB and BM was performed, a relative reduction in B220-positive B cells and a corresponding increase in myeloid cells were observed in oc/oc recipients compared to control recipients. No lineage skewing was observed in the RANK KO recipients compared to control recipients, in either PB or BM (Figure 2C and D).

Reduction in number of phenotypic but not in functional HSCs in oc/oc and RANK KO recipients

The number and function of HSCs in the BM of oc/oc, RANK KO and control recipients were analyzed by FACS and competitive secondary repopulation experiments. FACS analysis of the BM cells revealed a slight but signif-

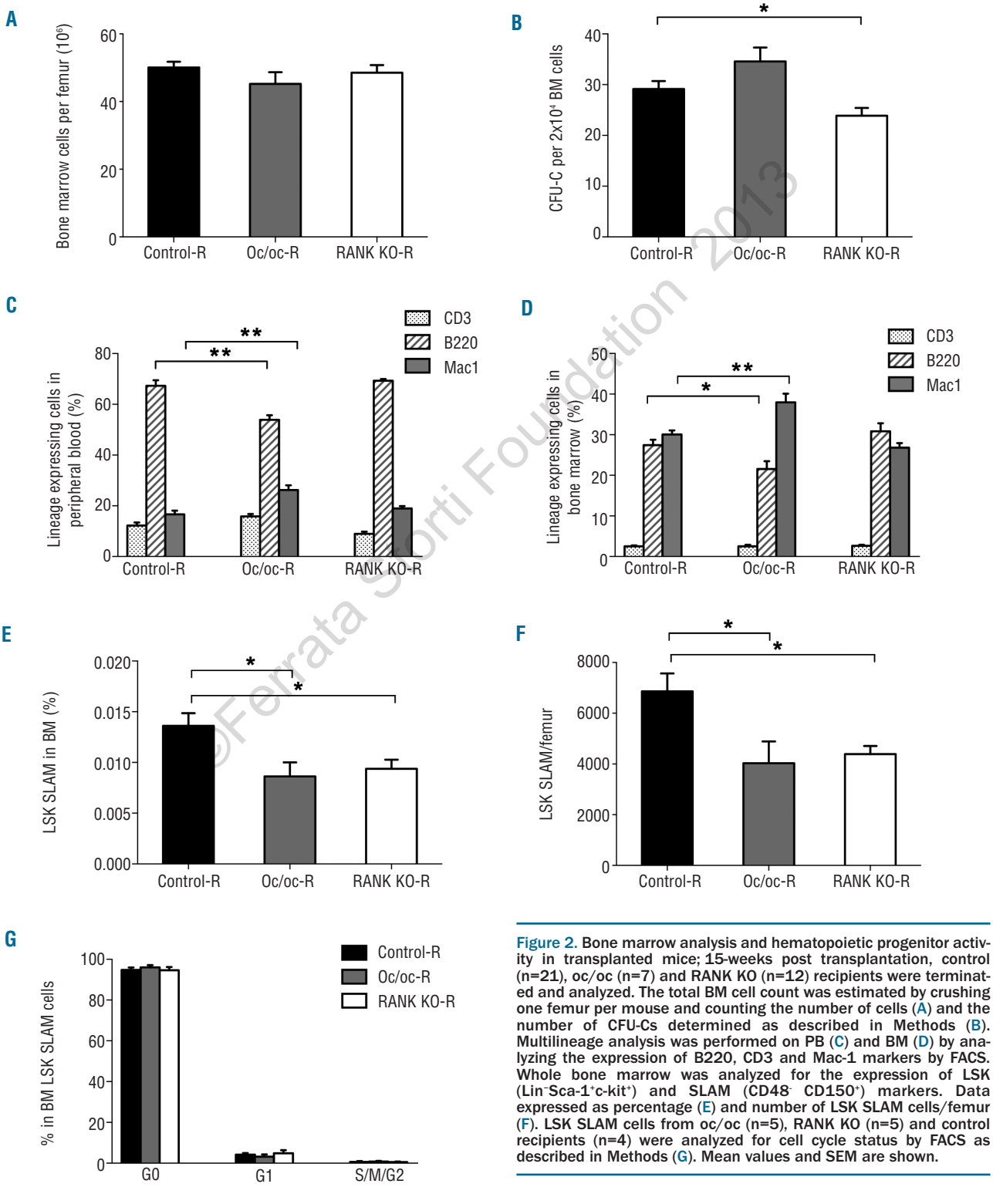

Figure 2. Bone marrow analysis and hematopoietic progenitor activity in transplanted mice; 15-weeks post transplantation, control (n=21), oc/oc (n=7) and RANK KO (n=12) recipients were terminated and analyzed. The total BM cell count was estimated by crushing one femur per mouse and counting the number of cells (A) and the number of CFU-Cs determined as described in Methods (B). Multilineage analysis was performed on PB (C) and BM (D) by analyzing the expression of B220, CD3 and Mac-1 markers by FACS. Whole bone marrow was analyzed for the expression of LSK ($Lin^-Sca\text{-}1^+c\text{-}kit^+$) and SLAM ($CD48^-$ $CD150^+$) markers. Data expressed as percentage (E) and number of LSK SLAM cells/femur (F). LSK SLAM cells from oc/oc (n=5), RANK KO (n=5) and control recipients (n=4) were analyzed for cell cycle status by FACS as described in Methods (G). Mean values and SEM are shown.

icant decrease in the number of immunophenotypically defined long-term HSCs, LSK SLAM cells, compared to controls (Figure 2E and F). To assess the cell-cycle status of this primitive hematopoietic population, LSK SLAM positive BM cells of the three groups were analyzed by FACS. No significant difference was found between the groups with regards to cell cycle profile. The proportion of cells in G0 was approximately 94-96% in all three groups (Figure 2G).

To compare the functional capacity of HSCs in the three groups, a competitive repopulation experiment was performed by transplanting 3 x 10^5 oc/oc, RANK KO or control recipient BM cells together with 2 x 10^5 WT competitor BM cells into secondary recipients. No functional disadvantage of stem cells from oc/oc or RANK KO recipients was observed when compared to control recipients in this setting (Figure 6 A-D).

Discussion

The HSC niche is a complex environment involving many different regulators. One key regulator is the osteoblast but recent findings have also highlighted the essential roles of endothelial and perivascular mesenchymal progenitors for maintenance of hematopoiesis.[13,31] Besides the tightly coupled relationship between the osteoblast and the osteoclast, the direct role of the osteoclast in the regulation of the fully developed hematopoietic niche in the adult animal is still controversial and needs to be fully clarified.

One approach used to investigate the role of osteoclasts for hematopoiesis has been to inhibit these cells pharmacologically with bisphosphonates. A recent publication by Lymperi and co-workers showed that when osteoclast function was blocked by injecting mice with bisphosphonate alendronate, HSC number was reduced.[23] However, another report stated that administration of the same compound resulted in expansion of HSCs and in an up-regulated mobilization of these cells to the circulation in response to G-CSF.[19] A third group reported that the bisphosphonate zolendronic acid did not affect HSC number even though it induced osteopetrosis.[32] Thus, this experimental approach has yielded contrasting results.

Another way to delineate the role of osteoclasts for hematopoiesis has been to examine the hematopoietic compartment in different mouse models of osteopetrosis, a group of diseases characterized by either a lack of osteoclasts or by osteoclasts with a defective function. Analyzing three such models, the op/op, c-Fos-deficient and RANKL (Receptor Activator of Nuclear Factor Kappa B ligand) deficient mice, Miyamoto and co-workers

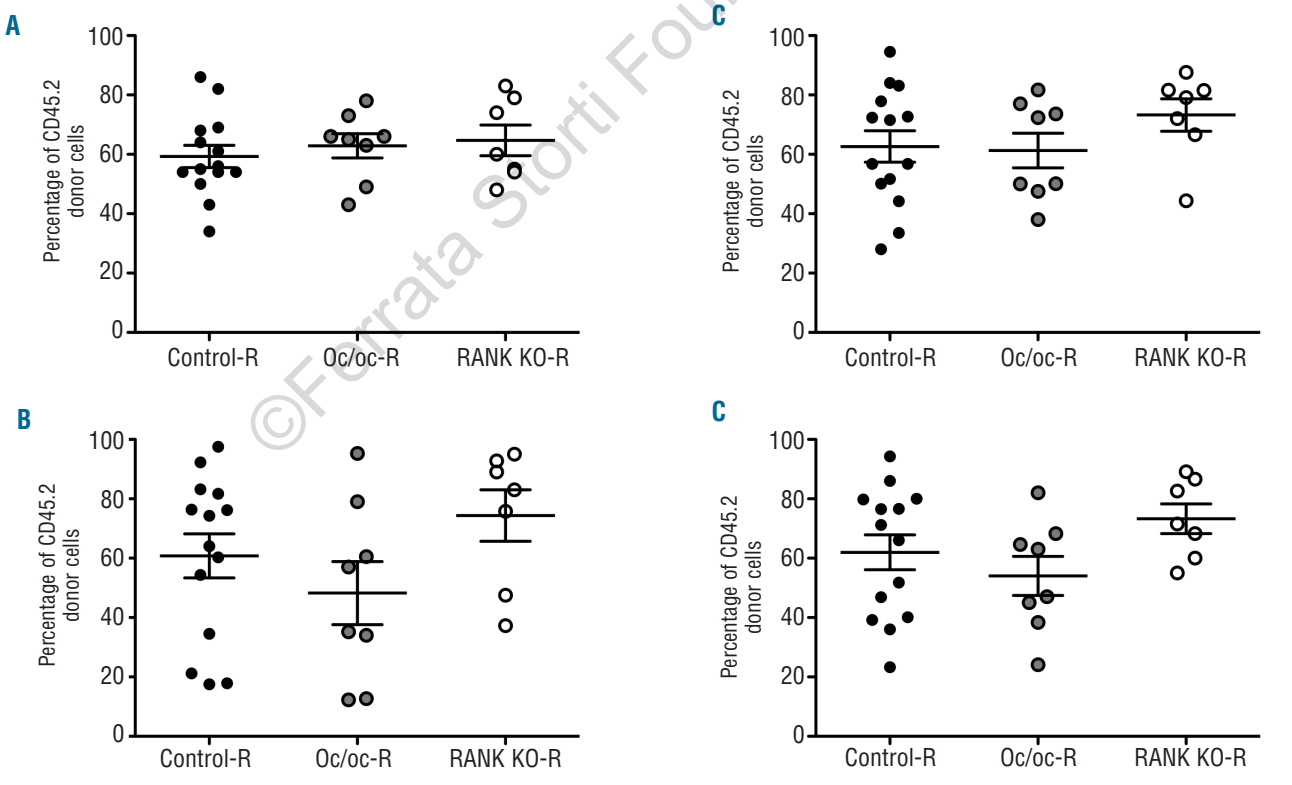

Figure 3. Long-term repopulating capacity of stem cells in oc/oc and RANK KO recipients does not differ from that of control recipients as analyzed by secondary competitive transplantation. 2x10^5 (CD45.1/2) fresh competitor BM cells were transplanted intravenously into lethally (950 cGy) irradiated wild-type (CD45.1) recipients with 3x10^5 thawed bone marrow cells obtained from control, oc/oc and RANK KO recipients (CD45.2), respectively. Bone marrow from three primary recipients were pooled and transplanted into 3 secondary recipient mice. Engraftment was determined by analyzing the percentage of CD45.1/CD45.2 expressing cells in secondary control (n=14), oc/oc (n=8) and RANK KO (n=7) recipients in peripheral blood after six (A), 13 (B) and 20 (C) weeks and in the bone marrow after 20 weeks (D). Individual mice, mean values and SEM are shown.

reached the conclusion that osteoclasts are dispensable for hematopoietic stem cell maintenance and mobilization.[19] The mice in these experiments were 8-12 weeks old, thus not yet fully mature adult mice. In contrast, Mansour *et al*. investigated the role of osteoclasts in the developing animal through analysis of the severe oc/oc mouse model of osteopetrosis that is characterized by an increased number of osteoclasts that lack resorptive capacity. They found that osteoclasts are required for formation of the HSC niche via the induction of osteoblastic development.

The aim of the present study was to determine whether osteoclasts and/or the osteoclast resorptive function are required for the maintenance and function of HSCs in fully developed adult mice. To avoid the use of pharmacological agents and their potential off-target effects, we, like Miyamoto and co-workers, employed two murine models of osteopetrosis, the osteoclast-rich oc/oc strain mentioned above and RANK KO mice that are devoid of osteoclasts. As these mice die well before adulthood, we elected to establish chimeric adult animals by transplanting fetal liver HSCs from the respective osteopetrotic strain to adult wild-type mice.

Both oc/oc and RANK KO recipients developed a mild osteopetrotic phenotype, similar to that found in a recent study using the oc/oc recipient model.[28] As analyzed both *in vitro* and *in vivo*, the oc/oc recipients exhibited an increased number of osteoclasts, whereas the RANK KO recipients developed a severe reduction of osteoclasts, as was expected and consistent with the original phenotypes having increased number or no osteoclasts, respectively.[24,25] The bone resorption marker CTX-I was equally reduced in both recipient groups, and significant and similar increases in femoral cortical bone volume fraction and cortical thickness was observed in both mouse models. This is also in line with previously published data.[28]

When analyzing the hematopoietic phenotype of the transplanted recipients, bone marrow cellularity was unchanged and comparable to control recipients in both experimental groups. The progenitor cell level, as determined by CFU-C, was slightly reduced in RANK KO recipients but comparable to controls in the oc/oc recipients. Lymperi *et al*. observed an increase in CFU-C frequency in the bone marrow of alendronate-treated animals, but when the total number of CFU-C per tibia was calculated, no difference was found compared to controls, which indicates that the alendronate treatment reduced the total bone marrow cell count in their experiments.[23]

Lineage analysis of hematopoietic cells revealed a slight relative decrease in B cells and a relative increase in myeloid cells both in the peripheral blood and in the bone marrow of oc/oc recipients. This is consistent with the original analysis of hematopoiesis in the oc/oc mouse strain where a block in B-cell development was observed.[32,33] Ourselves and others have previously shown that this block in B lymphopoiesis can be reversed by transplantation of normal HSCs or dendritic cells to oc/oc mice.[26,34] It has also been demonstrated that the block in B-cell development is linked to the decreased resorptive activity of osteoclasts that affects the expression pattern of cytokines and growth factors, e.g. IL-7 by bone marrow stromal cells, and leads to impaired retention of B-cell progenitors in the bone marrow.[32] Consequently B-cell numbers could also be decreased in normal mice by treating them with a bisphosphonate, zoledronic acid.[32] In contrast, we did not find any change in lineage expression in the PB or BM of RANK KO recipients that have reduced numbers of osteoclasts but normal function.

When analyzing the BM cells phenotypically by FACS, a slight reduction in the amount of LSK SLAM cells in oc/oc and RANK KO recipients was observed as compared to control recipients. To find out if the reduction of phenotypic HSCs was associated with any changes in cell cycle profile, we performed a cell cycle analysis of LSK SLAM cells, but found no significant differences between the oc/oc, RANK KO and control recipient mice. Furthermore, when the HSCs from primary transplanted mice were transplanted in a secondary setting with wild-type competitor BM cells, we did not observe any impaired function of stem cells derived from oc/oc and RANK KO recipients as compared to Control recipients. The reason for the discrepancy between the results from FACS analysis of HSCs and the competitive repopulation experiment is not clear. However, it should be noted that the decrease in phenotypic HSCs was relatively small and we regard the results from the competitive secondary repopulation analysis as being more important than the FACS data in terms of assessing the stem cell compartment in the primary transplant recipients.

In terms of explaining the differences between our findings and those that we previously mentioned of Lymperi *et al*.,[23] several things stand out. First of all, the degree of suppression of resorption is difficult to compare, as measurement of a resorption marker was included only in our study. When looking at the suppression of TRAP 5b, it appears that the *in vivo* reduction in osteoclast number in the RANK KO recipient group of our study was a little larger than that observed in the alendronate treated mice (50% compared to approx. 30%). In terms of bone volume changes, these are not directly comparable due to differences in the bone compartment assessed, the length of the intervention, and the age of the mice. We transplanted mice older than 12 weeks and followed them for another 15 weeks while Lymperi *et al*. treated the mice with alendronate when the mice were relatively young and still undergoing development (2-3 months old).[35] While alendronate is a potent inhibitor of osteoclasts, it is also well known that it may affect other cells present in bone, such as macrophages and various cells of the osteoblastic lineage directly.[36,37] On the other hand, confounding effects on HSCs or other niche components in the two mouse models used in the present study cannot be ruled out completely. Hence, there are numerous differences between the studies that could explain the different outcomes and conclusions. However, our data correlate well with the previously cited findings of Miyamoto *et al*. who found that ablation of osteoclast-precursors and osteoclasts did not alter the hematopoietic compartment.[19]

In the severe oc/oc mouse model, the non-functional osteoclasts during development lead to impaired formation of osteoblasts and subsequently a defect in the formation of the osteoblastic niche and hematopoiesis.[1] If these mice are provided with functional osteoclasts, the hematopoietic niche and hematopoiesis are both restored.[1,26,38] The reversal of osteopetrosis and restoration of normal hematopoiesis is rapid and can be accomplished by infusion of high doses of normal hematopoietic cells provided this takes place neonatally (first day after birth).[38] However, if the delivery of cells is delayed only one week, reversal of the osteopetrotic phenotype, normalization of hematopoiesis and prolongation of survival are not

achieved.[26] This clearly supports the concept of the importance and dynamic influence of the osteoclasts during early development. It also underscores the complexity of the interactions between hematopoietic cells, osteoclasts and other niche components such as osteoblasts and their progenitors, and the careful considerations that need to be made in relation to the different types and developmental stages of the experimental models investigated.

In summary, in the present study we investigated how a reduction in osteoclasts or the abolishment of the osteoclast resorptive function affect HSCs and the maintenance of hematopoiesis in adult mice that were followed for more than three months after establishment of hematopoiesis derived from two different osteopetrotic mouse strains. Overall, the changes seen in the hematopoietic compartment were minimal, even though the mice in both models developed mild osteopetrotic phenotypes. Most importantly, we found that the HSC function as determined in a secondary competitive transplantation setting in both oc/oc and RANK KO recipients was comparable to that of control recipients. Thus, the conclusion from our experiments is that osteoclasts appear not to be crucial for stem cell maintenance and function in adult mice.

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Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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