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Title: Acute hematopoietic stress in mice is followed by enhanced osteoclast maturation in the bone marrow microenvironment

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Abstract

Osteoclasts are components of HSC niches, but their role as contributors to the HSC homeostasis and release are still controversial. We aimed to investigate whether an acute blood loss of 10% of total blood content, and the consequent intense hematopoiesis would affect osteoclast differentiation and activity. Isolated peripheral blood (PBL), spleen, and bone marrow (BM) cells from bones of hind limbs were investigated for the presence of specific subpopulations of osteoclast precursors – $B220^{-}CD3^{-}NK1.1^{-}CD11b^{-/low}CD115^{+}CD117^{+}$ cells in BM, and $B220^{-}CD3^{-}NK1.1^{-}Gr\text{-}1^{-}CD11b^{+}CD115^{+}$ cells in PBL and spleen, as well as the $RANK^{+}$ cycle-arrested quiescent osteoclast precursors (QOPs). Expression of osteoclastogenesis-related genes: CD115, RANK, and cathepsin K, and the potential of BM cells to form osteoclast-like cells *in vitro* and their activity *in vivo* were also evaluated**.** We observed an increase in spleen cellularity and myelopoiesis during 1 week following blood loss, without any significant effects on BM cellularity or BM myeloid precursors including cells with high osteoclastogenic potential. However, at 1 week post-bleeding, hematopoiesis significantly promoted the expression of cathepsin K, IL-34, and BMP-6. QOPs increased significantly in spleen 2 days following bleeding, while osteoclast activity remained unchanged up to 2 weeks post-bleeding. Osteoclast-dependent B-cell differentiation was affected at the pre-B stage of maturation in BM, while the LSK population expanded in BM and spleen after 2 days post-bleeding. Our data demonstrate that an acute blood loss promotes differentiation and maturation of osteoclasts at 1 week, but does not enhance osteoresorption *in vivo* up to 2 weeks of the recovery period, and identifies osteoclast differentiation as a consequent and

important event in establishing HSCs homeostasis following hematopoietic stress.

Keywords: Hematopoiesis, Hematopoietic stem cells, Hemorrhage, Osteoclasts

Introduction

Mature blood cells originate in the bone marrow (BM) by proliferation and differentiation of hematopoietic stem cells (HSCs) within distinct hematopoietic niches, local microenvironments within the BM tissue, which are defined by both their anatomy and function [1]. Fine tuning of the processes within the niches, as well as mechanisms of HSC regulation, such as quiescence or mobilization from BM to the circulation [2] involve activities and interactions of different cell types [3] – endothelial cells, CXCL12-abundant reticular cells (CAR) [4] and a discrete population of tissue resident macrophages – osteomacs [5]. Various cells at the endosteal surface of trabecular bone – osteoblasts of mesenchymal and osteoclasts of hematopoietic origin, physically support HSCs and provide signals that control their fate [2,6–16].

Osteoclasts, bone resorbing cells, differentiate from myeloid progenitors, due to a variety of factors, most notably macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) [17]. These ligands bind to corresponding receptors c-Fms/CD115 (M-CSF receptor) and RANK (RANKL receptor) [17] expressed on osteoclast precursors, and the BM microenvironment is highly supportive of osteoclastogenesis [18]. Recently, several distinct osteoclast precursor subpopulations have been identified – $B220^{-}CD3^{-}NK1.1^{-}CD11b^{-/low}CD115^{+}CD117^{+}$ cells by Jacquin et al. [19] in the BM, and $B220^{-}CD3^{-}NK1.1^{-}CD11b^{+}Ly6C^{hi}CD115^{+}Ly6G^{-}CD117^{int}$ by Jacome-Galarza et al. [20] in the spleen and peripheral blood – both with a

very high potential to generate osteoclasts *in vitro*. Muto A et al. [21] have identified a population of $RANK^+$ cycle-arrested quiescent osteoclast precursors (QOPs) that circulate in blood and reach the bone. Their lifespan is between 6-8 weeks [22], while Interleukin (IL)-34, a newly discovered alternative ligand for CD115 produced by vascular endothelial cells, helps in their maintenance [23], especially in the spleen.

The exact role and mechanisms of mature osteoclasts as contributors to the HSC release from the niches are somewhat controversial. For the most part it seems they ease the release of HSCs into circulation [2,24–27] by secreting enzymes which mediate the cleavage of CXCL12/SDF-1 and other molecules responsible for the anchorage of HSCs in the niche. It has been shown that the inhibition of osteoclast function reduces the number of BM HSCs *in vivo* [28,29] and increases the number of HSCs in the spleen [30]. Others have, however, reported that osteoclasts are either dispensable for HSC maintenance and mobilization [31–33], or that they affect the HSC niche only indirectly, through an effect on osteoblast differentiation [25], while at the same time actively modulating B-cell development [34]. Taken together, this indicates that osteoclasts are important for the homeostasis of the BM microenvironment.

BM is not the only site for hematopoiesis in mice, resulting from development and trafficking of HSCs in non-marrow tissues during embryogenesis. Shortly before birth HSC migrate from the fetal liver to the BM, while the number of hematopoietic progenitors in the spleen increases. This establishes the spleen as a hematopoietic organ in adult mice, albeit at low levels [35], because the spleen contains very few primitive long-term reconstituting HSCs [36,37]. Its microenvironment supports development of erythroid cells [38], later stages of myeloid and dendritic cells, as well as BM-derived monocyte precursors [39], but, in physiological conditions, it is not conducive to osteoclastogenesis [40,41]. After an hematological stress in adult mice,

both BM and spleen niches are activated and stimulate HSC proliferation [22] to maintain the RBC count and oxygenation homeostasis [42]. The main regulator of RBC production is erythropoietin (Epo), produced mostly by kidneys, which serves as a good estimator of the extent of erythropoietic activity [43].

Since the effects of enhanced hematopoiesis on proliferation and differentiation of discrete osteoclast precursor subpopulations and osteoclast activity have not been fully elucidated, we hypothesized that the intense hematopoiesis following blood loss would also affect cells of the osteoclast lineage. To test this hypothesis, we subjected mice to an acute blood loss (10% of total blood volume) and monitored the indicators of hematopoietic homeostasis by determining the changes in populations of $Lin^{-}Sca1^{+}c\text{-}kit^{+}$cells, macrophages, and B-cell precursors, at the same time assessing the phenotype of discrete osteoclast precursors at several time points within the first follow-up week, and the activity up to 2 weeks following blood loss.

Materials and Methods

Mouse model of acute blood loss

All experiments were performed on female 10-12 week-old C57BL/6 mice. The animals were housed at the Animal Care Unit of the Institute for Brain Research, Zagreb. Maintenance of animals and all experimental procedures strictly followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal protocols were approved by the Ethics Committee of the University of Zagreb School of Medicine (Zagreb, Croatia). Animals were randomly distributed into six groups (control and bled groups – each group was bled only once at 12h, 24h, 48h, 72h or 1 week before sampling, n=3). The experiments were repeated at least three times. Mice were weighed to determine their approximate total blood content and then anesthetized with 3-bromoethanol (Sigma-Aldrich, Milwaukee, MI, USA) i.p. according to the manufacturer's instructions. The bleeding was performed by introducing a capillary glass tube (Lancer, Brunswick company, USA) at the medial canthus of the orbit to access the retro-orbital plexus [44], and 10% of the calculated blood volume was drawn. Mice were given appropriate fluid replacement. Samples for blood analysis were collected into BD microtainer K2E tubes containing EDTA. The number of reticulocytes and red blood cells were measured using the Sysmex XT-2000i Automated Hematology Analyzer (Sysmex America Inc., Lincolnshire, IL, USA).

In vitro cell culture

BM cells for osteoclast-like (OCL) cell differentiation were flushed out from femoral and tibial medullar cavities and prepared as previously described [45,46]. Non-adherent cells were harvested and plated into 96-well plates at a density of 0.18×10^6/well in 0.2

mL/well of α-MEM/10% FBS supplemented with 20 ng/mL (rm)M-CSF and 30 ng/mL rmRANKL (R&D Systems). At day 3 following plating cells were fixed and OCLs were identified by staining with a commercially available kit (Sigma-Aldrich), as tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells. Using light microscopy, cells with three or more nuclei were counted as OCL [45].

Flow cytometry

Isolated BM, spleen and peripheral blood cells were stained using commercially available anti-mouse lineage mixture kit (Alexa fluor 488 conjugated anti-CD3, anti-CD11b, anti-CD45R, anti-Ly6C/G and anti-TER119) (Invitrogen, Invitrogen corporation, CA, USA) in combination with PE-conjugated anti-Sca-1 and APC-conjugated anti-c-kit for identification of primitive HSCs. Identification of osteoclast progenitors was performed by using FITC-conjugated anti-mouse CD45R, CD3 and NK1.1; anti-mouse CD115-biotin conjugated in combination with streptavidin-PE conjugate, anti-mouse CD11b-Pe-Cy7 and anti-mouse CD117-APC or anti-mouse Gr-1 APC, also anti-mouse CD115-biotin in combination with streptavidin-FITC conjugate and anti-mouse RANK-PE antibodies. Anti-mouse-F4/80 PE and anti-mouse CD11b-Cy-7 were used for identification of monocyte/macrophage precursors. As an erythroid cell marker, anti-mouse-TER119 PE was used. Identification of B-cell precursors at different stages of development was performed by using anti-mouse IgM-FITC, anti-mouse CD43 PE, anti-mouse CD19 PeCy-7 and anti-mouse B220-APC. Unless otherwise indicated, the antibodies were purchased from eBioscience (Affymetrix, San Diego, CA, USA). Cells were analyzed for immunophenotypical enumeration of several specific cell populations, using dual laser FACSCalibur II (Beckton Dickinson, San Jose, CA, SAD) and CellQuest software (Becton Dickinson Immunocytometry Systems).

Gene expression analysis

Total RNA was extracted from isolated BM, spleen and homogenized bones (tibia) using TRI reagent (Applied Biosystems, Foster City, CA, USA) and reversely transcribed (1µg) to cDNA and amplified (20 ng/well) by quantitative (q)PCR using an ABI Prism 7500 Sequence detection system (Applied Biosystems) in a 25µL reaction volume [46]. Commercially available TaqMan Gene Expression Assays for osteoclast-specific genes: cFms *(Csf1r; Mm00432689_m1),* RANK (*Tnfrs11a;* Mm00437135_m1), and catepsinK *(CtsK; Mm00484039_m1);* and osteoblast-specific genes: runt-related transcription factor *(Runx2; Mm00501580_m1)*, osterix (Osx, *Sp7,* Mm00504574_m1), osteocalcin (OC, *Bglap1;* Mm03413826_m1), bone-sialoprotein(BSP, *Ibsp*, Mm00492555_m1), CXCL12/SDF-1 (*Sdf1* Mm00445553_m1), and bone morphogenetic protein (BMP)-6 (Mm01332882_m1); as well as IL-34 (Mm01243248_m1) were purchased from Applied Biosystems. The following primers for β-actin were used, forward: CAT TGC TGA CAG GAT GCA GAA, reverse: GCT GAT CCA CAT CTG CTG GA. Comparative quantification algorithms $\Delta\Delta$Ct, $2^{-\Delta\Delta Ct}$ formula, was used to express fold differences of the gene expression. Ct for the gene of interest in the test sample and the calibrator sample (control) were adjusted in relation to β-actin (housekeeping gene). Data are expressed as mean± standard deviation (SD) of the representative experiment, and SD values present variability between the technical replicates within a single experiment. The methodological studies of quantitative PCR analysis suggest that the least difference in gene expression that could be reproducibly detected is around 100% [46,47] and therefore we assume that a difference of 100% or more in gene expression, repeating through all experiments, is biologically significant.

Enzyme-linked immunosorbent assay (ELISA)

Blood samples, 500μL per mouse, were allowed to clot for 3 hours at room temperature before centrifuging for 20 min. at 2000 g. The serum was removed and stored at -20°C. Concentrations of serum erythropoietin (Epo) and mouse cross-linked C-telopeptide of type I collagen (CTX-I) were determined using commercially available ELISA kits (Quantikine Immunoassay, R&D systems, Minneapolis, MN, USA and Cusabio Biotech Co., Ltd., China, respectively) according to the manufacturers' directions.

Histology and Histomorphometry

Serial sections of mouse femora were prepared and stained for tartrate-resistant acid phosphatase (TRAP) activity as previously described [47]. Osteoclasts were identified as red multinucleated cells adjacent to the bone surface, and were counted in the whole diaphyseal areas, 1 mm distant from the epiphyseal plate. The total number of osteoclasts was normalized to the measured bone perimeter and expressed as the number of osteoclasts per millimeter of bone perimeter.

Statistical analysis

All experiments were performed at least three times. Numerical data for blood parameters (reticulocytes, erythrocytes and serum Epo levels), gene expression analysis and cell culture are expressed as mean±standard deviation (SD). Statistical analysis of the group difference, each time point versus control, was performed by unpaired two-tailed Student's test with Bonferroni correction for multiple testing giving the significance at $p \leq 0.01$ (calculated for $\alpha=0.05$ and total number of five comparisons – for each of the five experimental groups vs control). Statistical analyses were performed using MedCalc for Windows, version 13.0 (MedCalc Software, Ostend, Belgium).

Results:

Reduction by 10% of total blood volume significantly stimulates murine erythropoiesis

Mice were subjected to an acute blood loss of 10% of total blood volume estimated per mouse weight, at several time points up to one week, and erythropoietic parameters were assessed. The number of reticulocytes increased in peripheral blood (PBL) samples reaching significant difference at 1 week following bleeding compared to control ($p \leq 0.01$) **(Fig. 1a)**. In parallel, the number of erythrocytes slightly decreased, with full recovery at 1 week **(Fig. 1a)**. To further confirm the induction of erythropoiesis, we measured the concentration of serum Epo and found that it was significantly increased at 24h of the recovery period ($p \leq 0.01$) **(Fig. 1b)**. Flow-cytometric analysis of BM cells showed an approximately 1.4 fold increase of absolute number of $TER119^+$ erythroblasts, nucleated erythrocyte precursors, starting at day 3, and reaching significant difference at day 7 post-bleeding ($p \leq 0.01$) **(Fig. 1c)**. Moreover, there was a 5-fold increase in the absolute number of $TER119^+$ cells in the spleen at day 3, with significant difference at day 7 post-bleeding compared to control ($p \leq 0.01$) **(Fig. 1c)**.

Enhanced hematopoiesis expands murine LSK cells and alters the number of macrophages

To determine the changes of specific cell populations within BM and spleen microenvironment, we performed flow-cytometric analysis of BM, spleen and PBL samples. We observed no perceptible changes of total BM cellularity, while there was an increase of total spleen cellularity in the follow-up period **(Fig. 2a)**. In parallel, primitive hematopoietic cells, the $Lin^-Sca\text{-}1^+c\text{-}kit^+$ (LSK) population, expanded significantly by approximately 1.5 fold in BM at 48h to 72h compared to control

(p≤0.01). In spleen, the expansion of the LSK population started at 24h by 2.5 fold and significantly peaked at 1 week following blood loss (p≤0.01) **(Fig. 2b)**. Enhanced erythropoiesis affected the monocyte population, observed as an average 1.4 fold increase of $F4/80^{+}CD11b^{+}$ cells at 1 week post-bleeding in spleen and approximately 1.8 fold reduction in PBL starting at day 1, without significant changes in BM **(Fig. 2c)**.

Blood loss modifies the number of murine osteoclast precursors and B-lymphoid cells

We assessed the effects of blood loss on distinct subpopulations of osteoclast precursors in BM, spleen and PBL. There were no perceptible effects on the osteoclast precursor population ($B220^{-}CD3^{-}NK1.1^{-}CD11b^{-/low}CD115^{+}CD117^{+}$) in BM, **(Fig. 3a)**, spleen and PBL ($B220^{-}CD3^{-}NK1.1^{-}Gr\text{-}1^{-}CD11b^{+}CD115^{+}$) **(Fig. 3a).** We found that the number of QOPs ($CD11b^{-}Gr\text{-}1^{-}RANK^{high}CD115^{low}$) in spleen and PBL were increased during the entire follow-up period, reaching significant difference at days 2 to 7 in spleen, while it increased only transiently in the BM **(Fig. 3b)**.

Given the close association between B-lymphoid and osteoclast lineages [34,48,49], we also monitored the populations of B-cell precursors following blood loss. The number of pre-proB cells ($B220^{+}IgM^{-}CD43^{+}CD19^{-}$) in spleen showed a small increase during the post-bleeding period, with no changes in BM **(Fig. 4a).** The pro-B cell ($B220^{+}IgM^{-}CD43^{+}CD19^{+}$) population in BM was reduced after 48h, with a transient increase in spleen **(Fig. 4b).** Finally, the population of pre-B ($B220^{+}IgM^{-}CD43^{-}CD19^{+}$) cells was significantly reduced in BM at days 3 to 7 following blood loss (p≤0.01), with transient changes in spleen **(Fig. 4c).** The population sizes in PBL for all evaluated B-cell precursors were discrete, and no perceptible changes were observed (data not shown).

Alteration of osteoclast and osteoblast specific markers in murine BM following blood loss

To assess the markers of osteoclast differentiation and maturation, we investigated the expression of osteoclast-specific genes: CD115, RANK and CtsK, and the expression of IL-34. Only the expression of CtsK, an enzyme abundantly expressed in mature osteoclasts, was highly up-regulated toward the follow-up end point **(Fig. 5a)**. The expression of IL-34 was transiently down-regulated at 24h but then highly up-regulated at 1 week following blood loss **(Fig. 5a)**.

As the function and maturation of osteoclasts and osteoblasts is tightly coupled, we also evaluated the expression of osteoblast-specific genes: Runx2, Osx, BSP, OC and the expression of BMP-6. The expression pattern for Runx2 showed no changes throughout the evaluated period, while the most obvious up-regulation was observed for Osx (a transcription factor essential for osteoblast differentiation), although BSP, OC (markers of mature osteoblasts) and BMP-6 (an osteoinductive cytokine) followed a similar pattern **(Fig. 5b)**.

Blood loss alters the osteoclastogenic potential of murine BM cells *in vitro*

To assess the osteoclastic differentiation potential of bone marrow cells after an acute blood loss, we cultured BM cells in osteclastogenic conditions. The total number of $TRAP^+$ cells (≥ 3 nuclei/cell) was significantly lower ($p \leq 0.01$) at 24h and 1 week of the post-bleeding period compared with the control **(Fig 6a)**, but the osteoclasts were larger and contained significantly ($p \leq 0.01$) more nuclei/osteoclast **(Fig 6b)**. For *in vivo* evaluation of the osteoclast number and activity, we measured serum levels of mouse cross-linked C-telopeptide of type I collagen (CTX-I) and quantified tartrate-resistant

acid phosphatase (TRAP) positive cells in distal femoral sections. Serum levels of CTX-I showed no changes throughout the evaluated period, and the number of $TRAP^+$ cells present on the bone surface was comparable with control 2 weeks following blood loss.

Discussion

In this study, we have established a mouse model of acute blood loss to monitor alterations within bone cell lineages following enhanced hematopoiesis. We have confirmed previously reported modest effects of acute hemorrhage on total BM cellularity, and a consequent increase of extramedullary hematopoiesis [50]. We have demonstrated that hemorrhage does not influence BM myeloid precursors nor osteoclast precursor cells with a high osteoclastogenic potential. However, at 1 week, hematopoiesis significantly promoted the expression of markers of mature osteoclasts and osteoblasts, as well as IL-34 and BMP-6. To the best of our knowledge, we are among the first to give a comprehensive insight into the extent that mild hemorrhage, as a hematopoietic stress, has on osteoclast differentiation and activity, and demonstrated the promotion of osteoblast-coupled osteoclast maturation after 1 week of recovery, with unchanged bone resorption up to 2 weeks.

In our mouse model, we confirmed that blood loss of 10% total blood content is sufficient to significantly increase the number of reticulocytes and serum Epo levels, which are both good estimators of induced erythropoiesis [43,51]. This increase lead to an elevated number of $TER119^+$ cells in BM and spleen as early as 3 days after bleeding. In addition, the LSK population, as primitive hematopoietic stem cells, expanded both in BM and in spleen, as previously reported by Cheshier et al., [50]. Since the hematopoietic potential of HSCs in spleen differs from the BM [52], the expansion of LSK cells in spleen may be the result of an observed exaggerated extramedullary hematopoiesis, as well as a more frequent cell cycling in spleen [53]. The increased LSK population enables sufficient *de novo* differentiation, both in BM and spleen, to replenish lost blood cells.

We further investigated whether enhanced hematopoiesis alters a discrete population of $CD11b^{+}F4/80^{+}$, functionally diverse cells. In the BM, macrophages lodged throughout the marrow form erythroblastic islands, rings of attached erythroblasts to one or more macrophages, which support their proliferation and differentiation to mature blood cells. Resident macrophages, ie osteomacs, are important for bone remodeling and the maintenance and release of HSCs, as their depletion results in a loss of endosteal OBs and consequent HSC mobilization [5]. In spleen, among other roles, macrophages control the retention and trafficking of B lymphocytes in the marginal zone [54]. Interestingly, the $CD11b^{+}F4/80^{+}$ population of myeloid precursors in BM was not altered until 1 week post-bleeding, and the total number in spleen increased during the entire follow-up period. It can only partially be explained by an existence of a reservoir of resident and undifferentiated pool of cells ready to be activated to facilitate a prompt immune response. In addition, we can speculate that a 10% blood loss due to increased demands for blood cells, provided the expansion of myeloid cells in spleen, as a recent study revealed that overexpression of erythropoietic regulators causes an expansion of myeloid cells in BM and spleen, and disrupts B lymphopoiesis [55].

Indeed, the hematopoietic response to bleeding affected B-lymphopoiesis, and resulted in a reduction in the number of pre-B stage maturation-lymphocytes, most evident after 3 days of the follow-up period. This is in line with described effects of Epo administration in mice [34,56]. Given such diverse and important roles of macrophages in BM and spleen hematopoiesis, we can speculate that the hematopoietic microenvironment changes the lineage commitment of HSCs in stress conditions by promoting myeloid and decreasing lymphoid differentiation, similarly to processes during aging [57].

Cells of the monocyte-macrophage cell line, the osteoclasts, closely interact with other

cells of the BM niche, and it was proposed that bone resorption, as part of bone remodeling, provides the space needed for enhanced hematopoiesis, cell maturation, and development within the BM cavity [56]. Similarly, several studies indicated a positive correlation between enhanced erythropoiesis and osteoclastogenesis [56,58]. However, Miyamoto et al., showed an increased HSC mobilization in osteopetrotic mice with a mutation in the M-CSF gene, and reduced HSC mobilization in osteoporotic mice deficient for osteoprotegerin, a decoy receptor for RANKL [33]. These results may be a reflection of different animal models and analysis techniques.

We analyzed changes of several osteoclast precursor subpopulations. In BM, the highest *in vitro* osteoclastogenic differentiation potential is contained within three discrete populations, among which the $B220^{-}CD3^{-}NK1.1^{-}CD11b^{-/low}CD115^{+}CD117^{+}$ shows the highest potential [19], but we show that enhanced hematopoiesis had little effect on this osteoclast subpopulation in BM. Further, phenotypically similar subpopulations with high osteoclastogenic potential, expressing CD115 and CD11b, and efficiently forming OCLs *in vitro* conditions [20], in spleen and PBL [20] were slightly reduced 3 days post-bleeding. Although described as osteoclast precursors, these populations can also generate mature functional macrophages and dendritic cells, beside osteoclasts, making inducing properties of BM and bone microenvironment crucial in defining the fate of these progenitors. Therefore, we further analyzed $RANK^{high}CD115^{low}$ quiescent osteoclast precursors (QOPs), a population able to differentiate into osteoclasts without cell-cycle progression [21,22,59]. In our model, the number of QOPs in all analyzed tissues transiently increased during the follow-up week with the most prominent increase in spleen after 2 days post-bleeding, indicating induced lineage committed differentiation. Although, we focused our study on osteoclasts and osteoclast precursors, they largely depend on the interactions with and

activity of osteoblasts [23]. We observed an increased BM expression of genes specific for osteoblast differentiation and function (OC, Osx, BSP), which is in line with the study by Lucas et al., [60] who have shown increases of the mineral appositional rate, osteoblast numbers, and serum concentration of osteogenic growth peptide following an acute blood loss in rats. In our model, at 1 week following bleeding, the expression of pro-osteoblastic growth factors BMP-6 in BM [61], and cathepsin K, a bone resorbing proteinase excreted by mature osteoclasts that mediates the release of HSCs from BM [24,26,27], were increased. In addition, IL-34, a newly discovered ligand for c-Fms, produced by vascular endothelial cells (mostly in spleen, but also in bone), has been proposed as a factor controlling QOP maintenance [40]. Alongside M-CSF it seems to be key for homing of QOPs to bone [23]. Supported by a recent study which confirmed that committed osteoclast precursors exit the BM, circulate via the bloodstream, and enter the bone sites specified for resorption [62], we may speculate of maturation and distribution of committed osteoclast precursors, at one week post-bleeding. We investigated *in vivo* activity of osteoclasts by measuring serum levels of C-telopeptide of type I collagen (CTX-I), as an indicator of the rate of bone resorption, and determined the number of TRAP positive cells adjacent to the bone surface up to 2 weeks. At 1 week post-bleeding the maturation of osteoclasts is evident by elevated expressions of cathepsin K, IL-34, and osteoblast maturation markers (OC, Osx, BSP) and BMP-6. Bone remodeling and the number of TRAP+ cells adjacent to bone were not changed up to 2 weeks.

Several previous studies reported results regarding the effect of bleeding on osteoclast differentiation and activity. Johnell et al., [63] showed enhanced number of OCs on rib metaphysis on day 3, returning to control levels on day 7, in rats, while Kollet et al., [24] presented an elevated number of TRAP+ OC in mice femora peaking at day 7, following

blood loss. The study of Moreau et al., [64] reported no change of bone parameters and CTX levels in Balb/c mice, demonstrating unchanged OC activity following bleeding, although in *in vitro* conditions the number of TRAP+ OC was enhanced versus control. By comparing these results, it is evident that the number of OC and activity depends on the murine model, bleeding protocols and techniques used in analysis. Moreover, a recent study of Shiozawa et al., [58] showed elevated levels of EPO in mouse sera following bleeding, which was confirmed in our study. They have focused their research on the effect of EPO administration on bone cells, and reported induced number of OC at week 2 and 4, during constant EPO treatment, and showed induced osteoclastogenesis which is not accompanied with enhanced osteoclast activity in *in vitro* conditions. Reported results show an elevated number of OC in bone tissue following administration of increasing concentration of EPO, and although the authors suggest a central role of EPO in bone remodeling following bleeding, it is important to stress that such results depend on the study approach. In our model of acute blood loss, we measured hematopoietic parameters including EPO levels in sera, which showed a peek at day 2, and decreased to homeostatic levels during 1 week, which is in contrast to the approach of increased concentrations of EPO administered by Shiozawa et al. Further, we alayzed *in vitro* and in *vivo* osteoclastogenesis and *in vivo* osteoclast activity, and to our knowledge, this is the first time an emphasis was made on discrete osteoclast precursor subpopulations, following bleeding. . .

In this context, the increased expression of osteoclast and osteoblast differentiation, maturation, and function markers, can suggest that after 1 week following blood loss the activities of osteoclasts and osteoblasts have reached a new homeostatic level, allowing for the establishment of hematopoietic homeostasis and preventing the induction of bone resorption.

As repetitive blood loss of around 10% total blood amount is typical for full-blood donations, future studies on models of chronic critical-amount bleeding are needed to evaluate the potential long-term effects on bone remodeling.

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phenylhydrazine-treated and Plasmodium-infected mice. *PLoS One*. 2012;7(9):e46101.

Figure Legends

Figure 1. Loss of 10% of total blood volume significantly stimulates murine hematopoiesis

Animals were subjected to acute hematopoietic stress by drawing 10% of total blood volume per mouse with various intervals between bleeding (1 week, 72h, 48h, 24h, 12h) and sampling. A) Hematological analysis of the number of reticulocytes and erythrocytes in peripheral blood samples. B) Serum erythropoietin concentration measured by ELISA. Data are presented as mean±SD. The representative experiment out of three independent experiments (3 animals per group) with similar results is presented. C) Flow cytometry analysis of bone marrow and spleen cells positive for the erythroid cell marker TER119. Bar graphs show absolute number of $TER119^{+}$ cells in bone marrow and spleen presented as mean±SD of three independent experiments. Samples were obtained from three mice per group. Group versus control comparisons were calculated using unpaired two-tailed Student's test with Bonferroni correction for multiple testing giving the significance at $p \leq 0.01$. Control (CTRL) denotes nonbled mice.

Figure 1

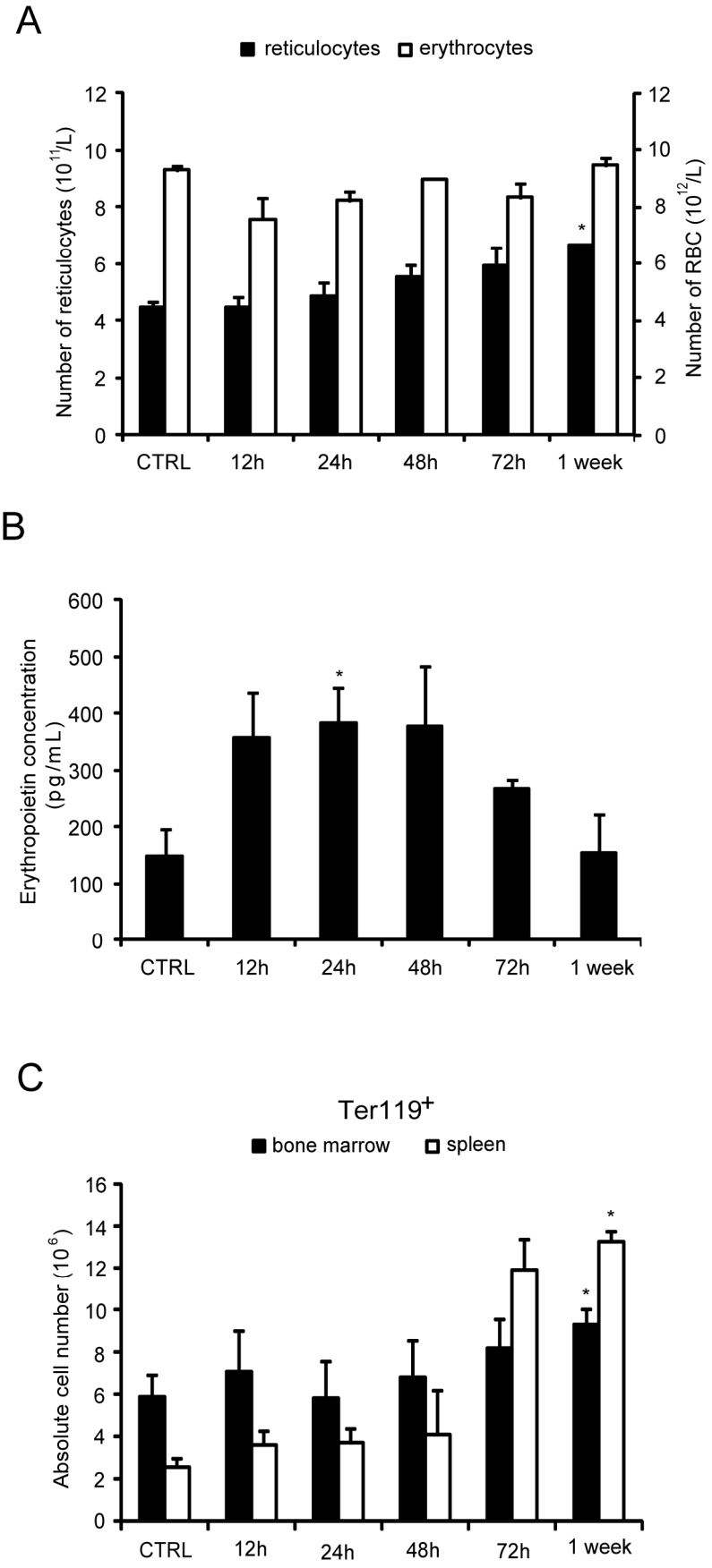

A
reticulocytes
erythrocytes
Number of reticulocytes (10^11/L)
Number of RBC (10^12/L)
0
2
4
6
8
10
12
CTRL
12h
24h
48h
72h
1 week
*
B
Erythropoietin concentration (pg/mL)
0
100
200
300
400
500
600
CTRL
12h
24h
48h
72h
1 week
*
C
Ter119+
bone marrow
spleen
Absolute cell number (10^6)
0
2
4
6
8
10
12
14
16
CTRL
12h
24h
48h
72h
1 week
*
*

Figure 2. Loss of 10% of total blood volume alters the number of murine LSK and myeloid cells

Flow cytometric analysis of freshly isolated bone marrow, spleen and blood samples. A) Changes of total cellularity of bone marrow and spleen B) Flow cytometry analysis of Lin^- $Sca\text{-}1^+c\text{-}kit^+$ (LSK) cells from bone marrow and spleen. C) Flow cytometry analysis of $F4/80^+CD11b^+$ myeloid progenitors in bone marrow, spleen, and blood.

Samples were obtained from three mice per group. Data are presented as mean±SD of three independent experiments. Control (CTRL) denotes nonbled mice. Bar graphs show absolute number of bone marrow, spleen and the percentage of the analyzed population in blood.

Figure 2

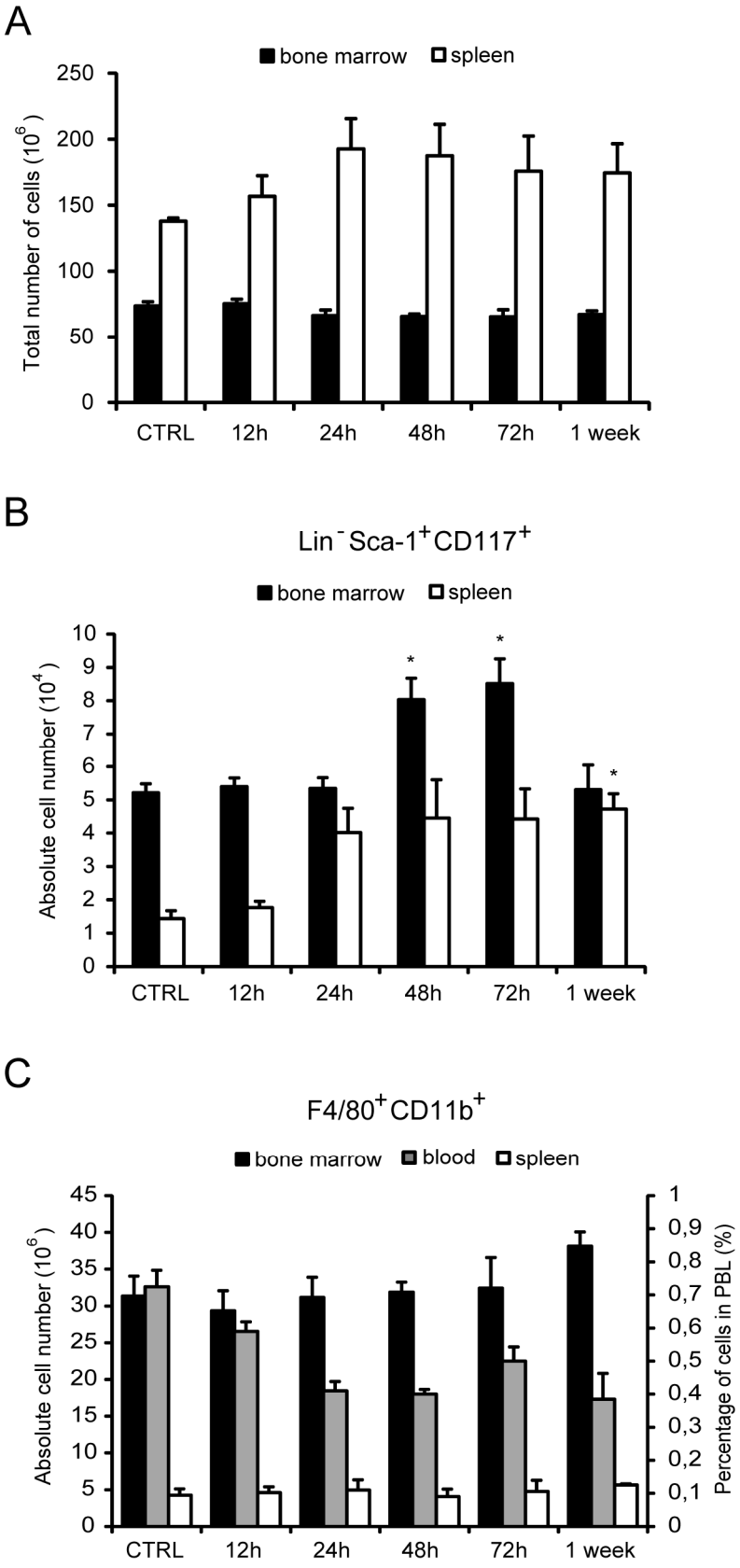

A
bone marrow
spleen
Total number of cells (10^6)
250
200
150
100
50
0
CTRL
12h
24h
48h
72h
1 week
B
$Lin^-Sca\text{-}1^+CD117^+$
bone marrow
spleen
Absolute cell number (10^4)
10
9
8
7
6
5
4
3
2
1
0
*
*
*
CTRL
12h
24h
48h
72h
1 week
C
$F4/80^+CD11b^+$
bone marrow
blood
spleen
Absolute cell number (10^6)
45
40
35
30
25
20
15
10
5
0
Percentage of cells in PBL (%)
1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
CTRL
12h
24h
48h
72h
1 week

Figure 3. Loss of 10% of total blood volume affects murine osteoclast precursor population size

Flow cytometric analysis of freshly isolated bone marrow, spleen and blood samples. A) Bar graphs show absolute number of bone marrow $B220^{-}CD3^{-}NK1.1^{-}CD11b^{-/low}CD115^{+}CD117^{+}$ and $B220^{-}CD3^{-}NK1.1^{-}Gr\text{-}1^{-}CD11b^{+}CD115^{+}$ cells in spleen and the percentage of cells in blood with high potential to generate osteoclast *in vitro*. B) Bar graphs present absolute number of bone marrow, spleen and the percentage of blood of $CD11b^{-}Gr\text{-}1^{-}RANK^{high}CD115^{low}$ cells.

Samples were obtained from three mice per group. Data are presented as mean±SD of three independent experiments. Control (CTRL) denotes nonbled mice.

Figure 3

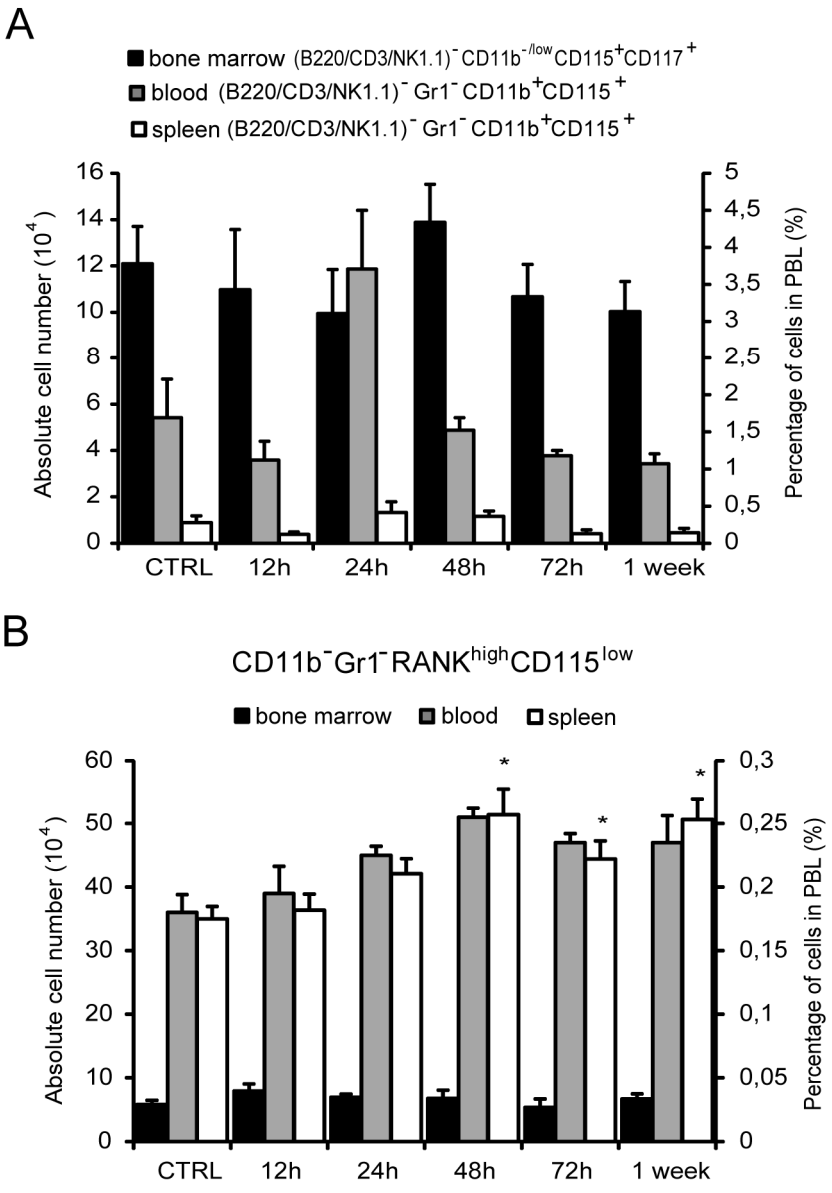
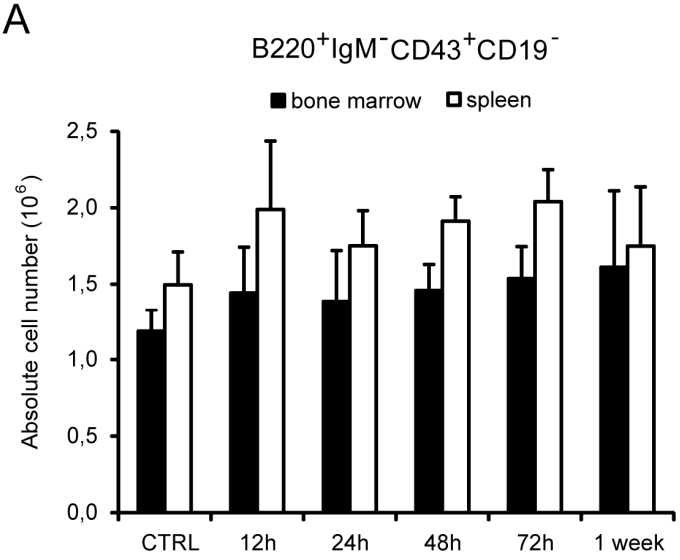

A
bone marrow (B220/CD3/NK1.1)⁻CD11b⁻/ˡᵒʷCD115⁺CD117⁺
blood (B220/CD3/NK1.1)⁻Gr1⁻CD11b⁺CD115⁺
spleen (B220/CD3/NK1.1)⁻Gr1⁻CD11b⁺CD115⁺
Absolute cell number (10⁴)
Percentage of cells in PBL (%)
0
2
4
6
8
10
12
14
16
0
0,5
1
1,5
2
2,5
3
3,5
4
4,5
5
CTRL
12h
24h
48h
72h
1 week
B
CD11b⁻Gr1⁻RANKʰⁱᵍʰCD115ˡᵒʷ
bone marrow
blood
spleen
Absolute cell number (10⁴)
Percentage of cells in PBL (%)
0
10
20
30
40
50
60
0
0,05
0,1
0,15
0,2
0,25
0,3
*
*
*
CTRL
12h
24h
48h
72h
1 week

Figure 4. Loss of 10% of total blood volume influences murine B-lymphopoiesis

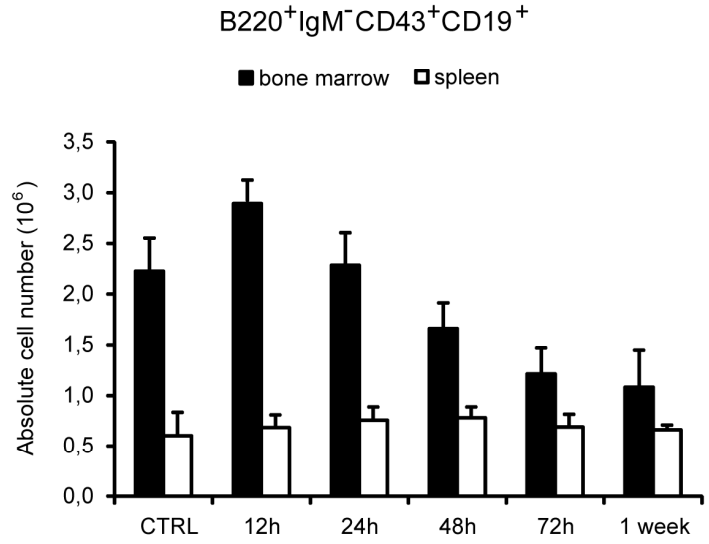
Flow cytometric analysis of freshly isolated bone marrow, spleen and blood samples. Bar graphs present absolute number of bone marrow, spleen and the percentage of blood cells of A) pre-proB ($B220^{+}IgM^{-}CD43^{+}CD19^{-}$), B) proB ($B220^{+}IgM^{-}CD43^{+}CD19^{+}$), and C) preB ($B220^{+}IgM^{-}CD43^{-}CD19^{+}$) cells. Samples were obtained from three mice per group. Data are presented as mean±SD of three independent experiments. Control (CTRL) denotes nonbled mice.

Figure 4

A


B220+IgM-CD43+CD19-
bone marrow
spleen
Absolute cell number (10^6)
2,5
2,0
1,5
1,0
0,5
0,0
CTRL
12h
24h
48h
72h
1 week

B


B220+IgM-CD43+CD19+
bone marrow
spleen
Absolute cell number (10^6)
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
CTRL
12h
24h
48h
72h
1 week

C

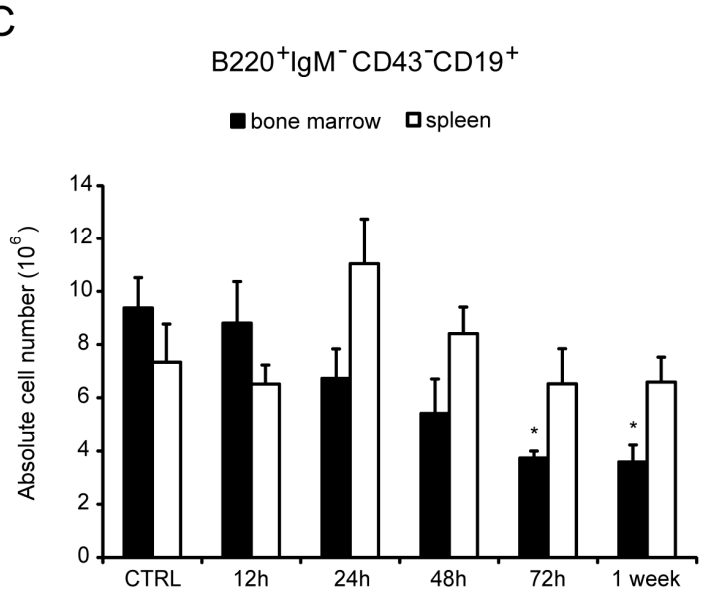

B220+IgM-CD43-CD19+
bone marrow
spleen
Absolute cell number (10^6)
14
12
10
8
6
4
2
0
*
*
CTRL
12h
24h
48h
72h
1 week

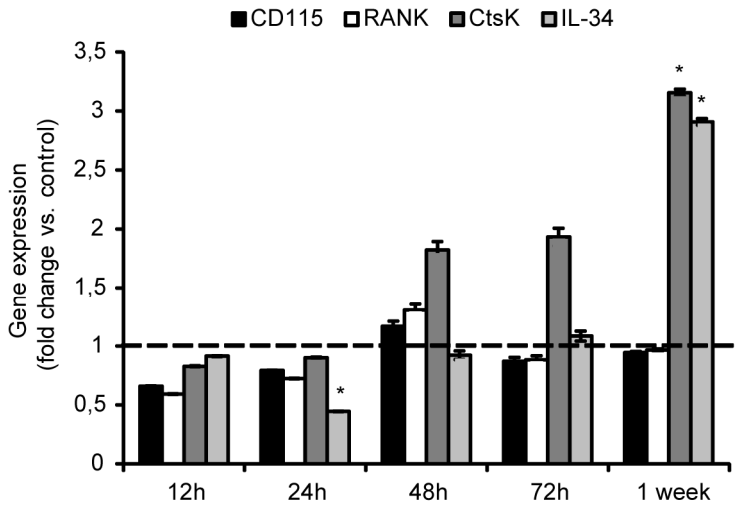
Figure 5. Blood loss promotes expression of murine osteoclast and osteoclast specific genes at one week following bleeding

Real-time RT-PCR analysis of the expression of osteoclast and osteoblast differentiation genes. A) Expression of cFms (CD115), receptor activator of nuclear factor κB ligand (RANK), cathepsin K (CtsK) and interleukin-34 (IL-34) in bone marrow. B) Gene expression patterns of Runx2, osterix (Osx), bone sialoprotein (BSP), osteocalcin (OC) and bone morphogenetic protein 6 (BMP-6) in bone marrow.

Data were normalized to β-actin. Fold changes were calculated using ΔΔCt method, and data are presented as mean±SD of PCR reaction technical duplicates. A relative quantification was considered significant at minimum two-fold change. Data are representative of those obtained for three mice in each group in three independent experiments.

Figure 5

A


CD115
RANK
CtsK
IL-34
Gene expression
(fold change vs. control)
3,5
3
2,5
2
1,5
1
0,5
0
12h
24h
48h
72h
1 week
*

B

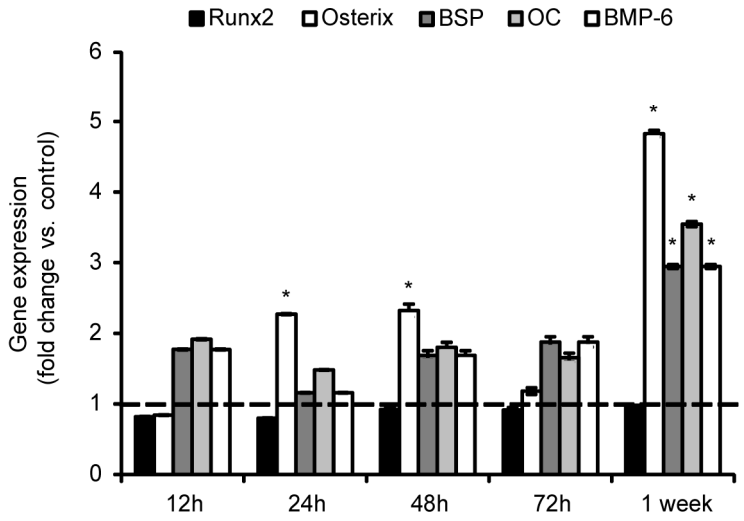
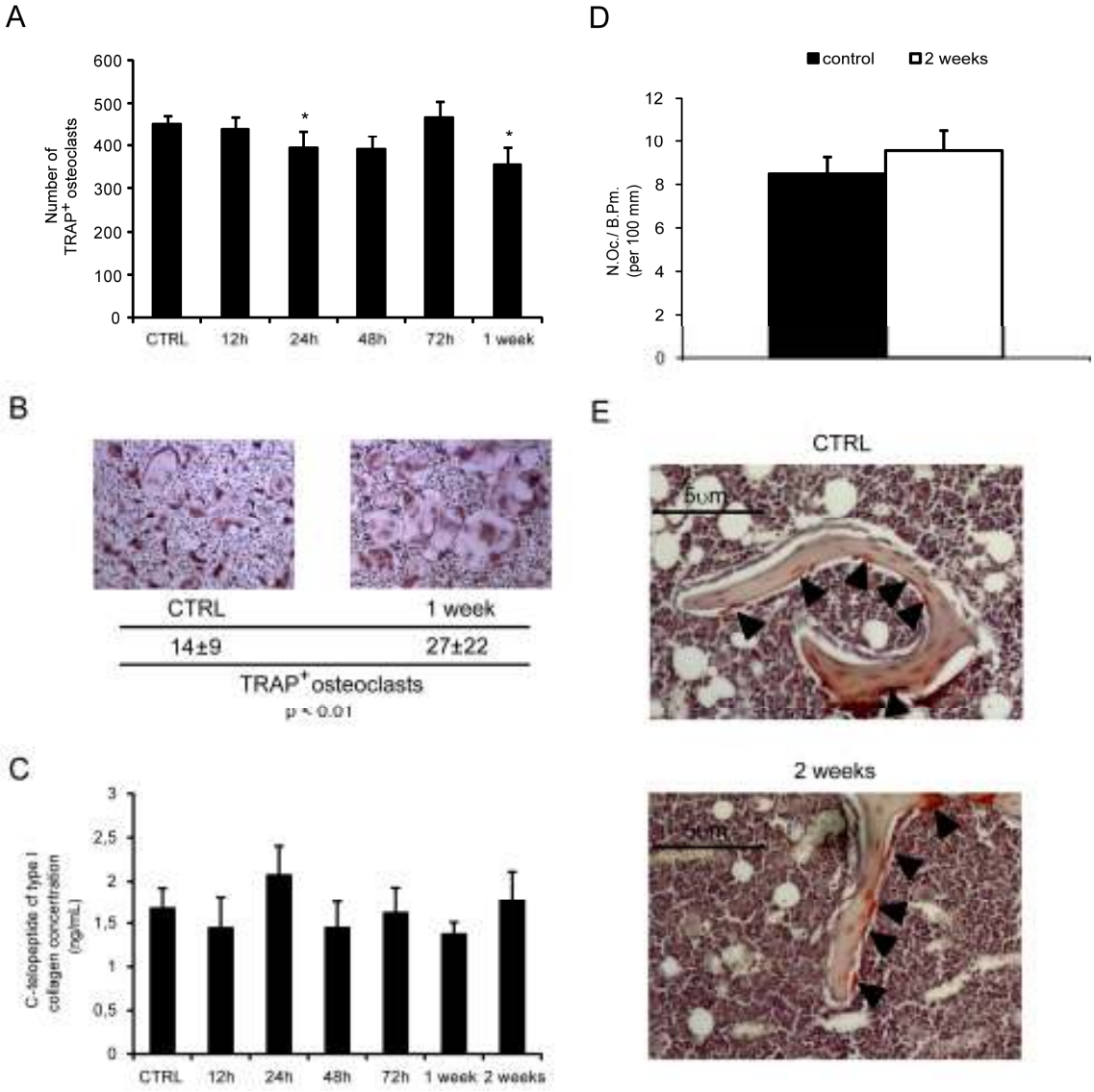

Runx2
Osterix
BSP
OC
BMP-6
Gene expression
(fold change vs. control)
6
5
4
3
2
1
0
12h
24h
48h
72h
1 week
*

Figure 6. Reduction of murine *in vitro* osteoclastogenic potential but not *in vivo* activity following blood loss

Osteoclastogenic potential, number and activity of BM cells *in vitro* and *in vivo*. Isolated bone marrow cells were cultured in the presence of macrophage colony-stimulating factor (M-CSF) for 24h, and nonadherent cells were subsequently plated with receptor activator of nuclear factor κB ligand (RANKL) and M-CSF for 3 days to generate tartrate-resistant acid phosphatase (TRAP)-positive osteoclast. Multinuclear TRAP+ cells with three or more nuclei were counted. Mouse sera were used for quantification of mouse cross-linked C-telopeptide of type I collagen (CTX-I) and TRAP+ cells were counted in femoral sections. A) Number of TRAP+ cells in bone marrow B) Representative light-microscopic images of cells expressing TRAP in bone marrow cultures with indicated average number of nuclei per osteoclast. Original magnification at 10×. C) Serum levels of CTX-I. D) Number of TRAP+ cells per bone perimeter. E) Representative light-microscopic images of TRAP stained osteoclasts, indicated with arrowheads, in distal sections of femora of untreated mice (left) and bled mice after 2 weeks following bleeding (right). Original magnification 20×.

Data are presented as mean±SD, and are representative of those obtained for three mice in each group in three independent experiments. Control (CTRL) denotes nonbled mice. Group versus control comparisons were calculated using unpaired two-tailed Student's test with Bonferroni correction for multiple testing giving the significance at $p \leq 0.01$.

Figure 6


A
Number of TRAP+ osteoclasts
600
500
400
300
200
100
0
CTRL
12h
24h
48h
72h
1 week
*
*
D
control
2 weeks
N.Oc./B.Pm. (per 100 mm)
12
10
8
6
4
2
0
B
CTRL
1 week
14±9
27±22
TRAP+ osteoclasts
p < 0.01
E
CTRL
50µm
2 weeks
50µm
C
C-telopeptide of type I collagen concentration (ng/mL)
3
2,5
2
1,5
1
0,5
0
CTRL
12h
24h
48h
72h
1 week
2 weeks