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

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Osteoclasts are components of hematopoietic stem cell (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, along with the consequent intense hematopoiesis, would affect osteoclast differentiation and activity. Isolated peripheral blood, 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 peripheral blood and spleen as well as the receptor activator of nuclear factor κ-B^+ cycle-arrested quiescent osteoclast precursors. Expression of osteoclastogenesis-related genes CD115, receptor activator of nuclear factor κ-B, and cathepsin K, the potential of BM cells to form osteoclast-like cells in vitro, and osteoclast activity in vivo were also evaluated. We observed an increase in spleen cellularity and myelopoiesis during week 1 following blood loss, without any significant effects on BM cellularity or BM myeloid precursors, including cells with high osteoclastogenic potential. However, at 1 week postbleeding, hematopoiesis significantly promoted the expression of cathepsin K, interleukin-34, and bone morphogenetic protein-6. Quiescent osteoclast precursors increased significantly in spleen 2 days following bleeding, whereas osteoclast activity remained unchanged up to 2 weeks postbleeding. Osteoclast-dependent B-cell differentiation was affected at the pre-B stage of maturation in BM, whereas the $Lin^-Sca\text{-}1^+c\text{-}kit^+$ population expanded in BM and spleen after 2 days postbleeding. Our data demonstrate that an acute blood loss promotes differentiation and maturation of osteoclasts at 1 week but does not enhance osteoresorption at 2 weeks postbleeding. Our data also identify osteoclast differentiation as a consequent and important event in establishing HSC homeostasis following hematopoietic stress. © **2014 ISEH - International Society for Experimental Hematology. Published by Elsevier Inc.**

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 (CARs) [4], and a discrete population of tissue resident macrophages called osteomacs [5]. Various cells at the endosteal surface of trabecular bone, namely 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 owing to a variety of factors, most notably macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κ-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 $B220^-CD3^-NK1.1^-CD11b^+Ly6C^{hi}CD115^+Ly6G^-CD117^{int}$

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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 and 8 weeks [22], and 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's release from the niches are somewhat controversial. It seems that they ease the release of HSCs into circulation [2,24–27] by secreting enzymes that mediate the cleavage of C-X-C motif chemokine 12/stromal cell-derived factor 1 (CXCL12/SDF-1) and other molecules responsible for the anchorage of HSCs in the niche. The inhibition of osteoclast function has been shown to reduce the number of BM HSCs in vivo [28,29] and to increase the number of HSCs in the spleen [30]. Others have, however, reported that either osteoclasts are 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.

Bone marrow is not the only site for hematopoiesis in mice, as evidenced by the development and trafficking of HSCs in nonmarrow tissues during embryogenesis. Shortly before birth, HSCs migrate from the fetal liver to the BM, and 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, and BM-derived monocyte precursors [39], but in physiologic conditions, the spleen is not conducive to osteoclastogenesis [40,41]. After an hematologic stress in adult mice, both BM and spleen niches are activated and stimulate HSC proliferation [22] to maintain the red blood cell count and oxygenation homeostasis [42]. The main regulator of red blood cell production is erythropoietin (Epo), which is produced mostly by kidneys and 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 also would 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 after blood loss.

Materials and methods

Mouse model of acute blood loss

All experiments were performed on female 10- to 12-week-old C57BL/6 mice. The animals were housed at the Animal Care Unit of the Institute for Brain Research (Zagreb, Croatia). 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 12 hours, 24 hours, 48 hours, 72 hours, 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) intraperitoneally. according to the manufacturer's instructions. The bleeding was performed by introducing a capillary glass tube (Lancer, Brunswick, GA) 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 ethylenediaminetetraacetic acid (EDTA). The number of reticulocytes and red blood cells were measured using the Sysmex XT-2000i Automated Hematology Analyzer (Sysmex America, Lincolnshire, IL).

In vitro cell culture

Bone marrow cells for osteoclast-like cell (OCL) differentiation were flushed out from femoral and tibial medullar cavities and prepared as previously described [45,46]. Nonadherent cells were harvested and plated into 48-well plates at a density of 0.4×10^6/well in 0.2 mL/well of α-minimal essenital medium (MEM)/10% fetal bovine serum supplemented with 20 ng/mL (recombinant mouse [rm]) M-CSF and 30 ng/mL rm RANKL (R&D Systems, Minneapolis, MN). 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 OCLs [45].

Flow cytometry

Isolated BM, spleen, and peripheral blood cells were stained using commercially available antimouse lineage mixture kit (Alexa fluor 488 conjugated anti-CD3, anti-CD11b, anti-CD45R, anti-Ly6C/G, and anti-Ter119) (Invitrogen, Carlsbad, CA) in combination with phycoerythrin (PE)-conjugated anti-Sca-1 and allophycocyanin (APC)-conjugated anti-c-kit for identification of primitive HSCs. Identification of osteoclast progenitors was performed by using fluorescein isothiocyanate (FITC)-conjugated antimouse CD45R, CD3, and NK1.1; antimouse CD115-biotin conjugated in combination with streptavidin-PE conjugate, antimouse CD11b-Pe-Cy7, and antimouse CD117-APC or antimouse Gr-1-APC; and antimouse CD115-biotin in combination with streptavidin-FITC conjugate and antimouse RANK-PE antibodies. Antimouse-F4/