Osteomyelosclerosis, anemia and extramedullary hematopoiesis in mice lacking the transcription factor NFATc2

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Citation: Bauer W, Rauner M, Haase M, Kujawski S, Arabanian LS, Habermann I, Hofbauer LC, Ehninger G, and Kiani A. Osteomyelosclerosis, anemia and extramedullary hematopoiesis in mice lacking the transcription factor NFATc2. Haematologica 2011;96(11):1580-1588. doi:10.3324/haematol.2011.042515

Online Supplementary Design and Methods

Flow cytometry

The following antibodies were used for the distinction of the different hematopoietic lineages: CD11b-phycoerythrin (PE), GR-1 (Ly-6C/G)-fluorescein isothiocyanate (FITC), and CD45R-FITC (Caltag Laboratories, San Francisco, USA); CD3-PE, CD4 (L3T4)-PE, CD8 (Ly-253-6,7)-PE, CD71-PE, Sca-1 (Ly-6A/E)-PE, Ter119-FITC, and IgG1-PE (BD Pharmingen, Palo Alto, USA); IgG2b-FITC (Beckman Coulter, Palo Alto, USA). A total of 10^5 blood or bone marrow cells or 8×10^5 spleen or liver cells were resuspended in phosphate-buffered saline (PBS) with 1% fetal calf serum (FCS) and incubated with 2 μL of the respective antibody mixtures for 20 min in the dark. When whole blood was used, red blood cells were lysed after the staining procedure using a commercial lysing solution (BD Biosciences) for 10 min at room temperature. Cells were washed and resuspended in 250 μL cell wash solution. Isotype-matched antibodies and unstained cells were used as negative controls. A total of 10,000 events were collected for each sample using a FACSCalibur flow cytometer (BD Biosciences, San Jose, USA) and analyzed using the CellQuest software (BD Biosciences, San Jose, USA). Dead cells were excluded by propidium iodide staining. Leukocyte subpopulations were defined as published elsewhere.[1,2]

Colony-forming assays

Single cell suspensions collected from the bone marrow, spleen, and liver were suspended in RPMI supplemented with 10% FCS and 1% penicillin/streptomycin and mixed with methylcellulose-based semi-solid (MethoCult) medium according to the manufacturer's protocol (Stem Cell Technologies, Vancouver, Canada). To assess the number of burst-forming units-erythrocytes (BFU-E), colony-forming units-granulocyte/macrophage (CFU-GM), CFU-granulocyte (CFU-G), and CFU-macrophage (CFU-M), 2×10^4 bone marrow cells, and 1×10^5 spleen or liver cells were cultured for 12 days in MethoCult GF M3434. The number of CFU-megakaryocytes (CFU-MK) was determined by culturing 2.2×10^6 bone marrow cells or 1.1×10^7 splenocytes for 10 days in MethoCult GF M4960.

Differentiation of megakaryocytes

Megakaryocytes were differentiated from $CD34^+$ cells isolated from the peripheral blood of G-CSF-mobilized healthy stem cell donors as described previously.[3] The study was approved by the institutional review board of the Medical Faculty of the Technical University of Dresden, and informed consent was obtained from the donors. Briefly, $CD34^+$ cells were positively selected using the CD34 Microbead kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured in Cellgro Stem Cell Growth Medium (CellGenix, Freiburg, Germany) in the presence of 100 ng/mL thrombopoietin for up to 14 days. The purity of $CD34^+$ cells was assessed by flow cytometry and routinely exceeded 95%. The megakaryocyte differentiation efficiency was analyzed for each experiment by morphological examination of cytospin preparations stained with May-Grünwald Giemsa as well as by flow cytometric analysis of the lineage-specific cell surface markers CD61 and CD42b. Where indicated, differentiation was performed in the presence of 0.1 μg/mL FK506.

Generation and assessment of osteoblasts and osteoclasts

Bone marrow cells were flushed out of femora and tibiae. For the generation of osteoclasts, 2×10^6 cells were cultured in 48-well plates and treated with 10 ng/mL M-CSF and 25 ng/mL RANKL (both from R&D Systems) for 7 days. Afterwards, cells were fixed and stained for TRAP using a commercially available kit (Sigma). Osteoblasts were generated by seeding 1×10^6 bone marrow cells per well of a 48-well plate and differentiating them in 5 mM β-glycerol phosphate and 100 μM ascorbate phosphate for up to 21 days. Alkaline phosphatase (ALP) activity and mineralization capacity were assessed as previously described. Proliferation of osteoblast progenitors was measured using a BrdU enzyme-linked immunosorbent assay (Roche) after 7 days of differentiation according to the manufacturer's instructions.

References

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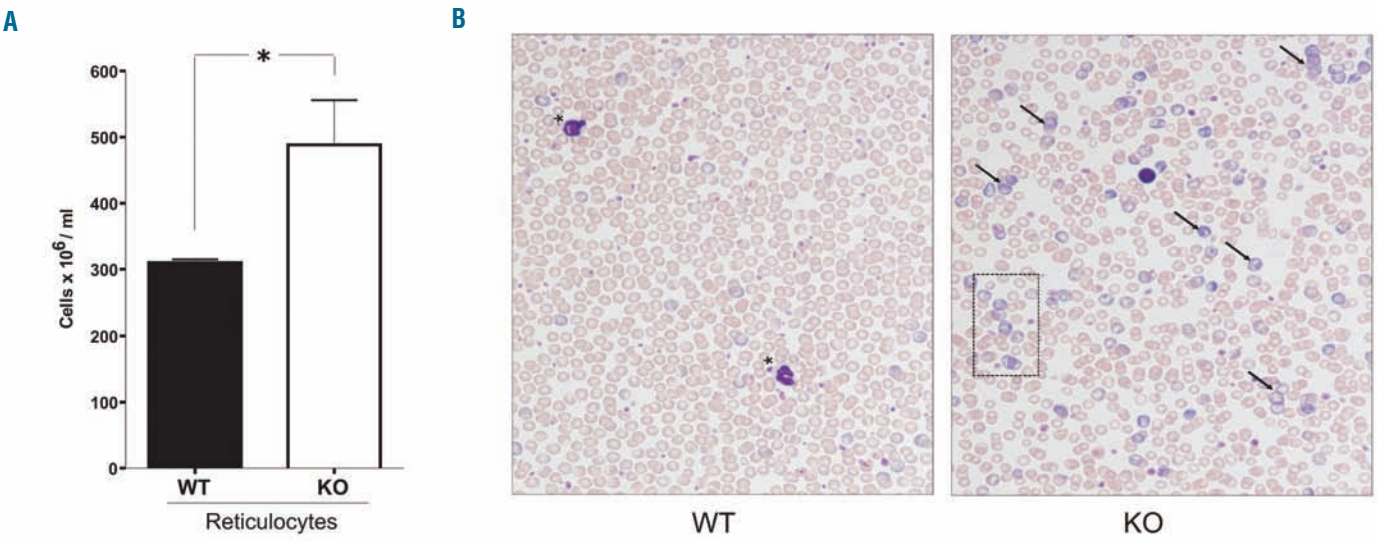
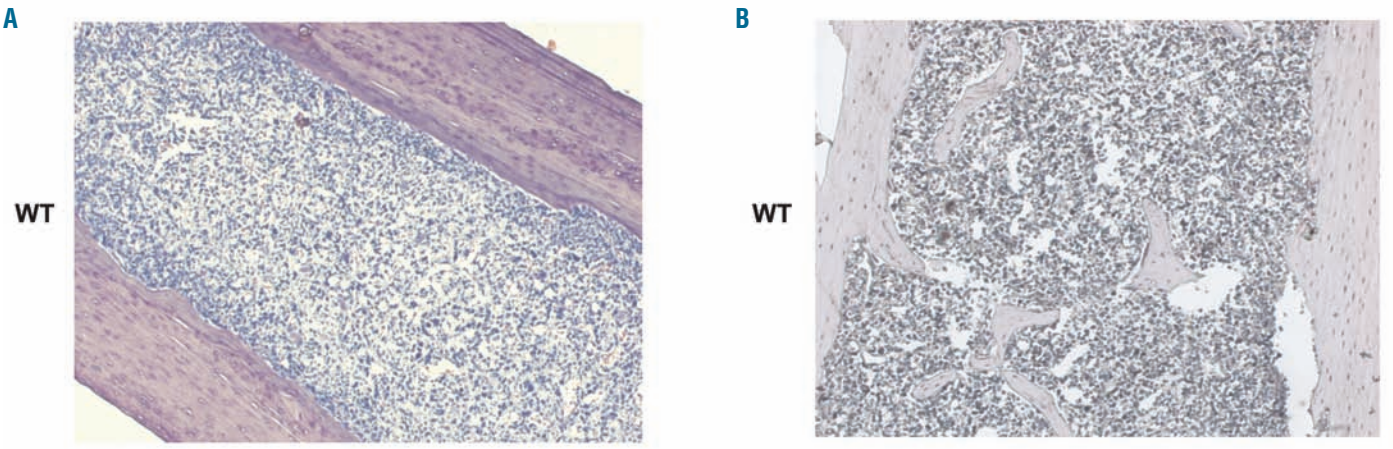
3. Kyttala S, Habermann I, Minami T, Ehninger G, Kiani A. Regulation of Down syndrome critical region 1 expression by nuclear factor of Activated T cells in megakaryocytes. Br J Haematol 2009;144(3):395-408.



Online Supplementary Figure S1. Reticulocytosis in aged NFATc2$^{-/-}$ mice. (A) Number of reticulocytes of aged (>12 months) NFATc2 knock-out (KO) *versus* wild-type (WT) mice. Results are expressed as means ± SEM of n=9-11 mouse pairs. *P<0.05. (B) Increased number of polychromatic reticulocytes (marked with arrows) in blood smears of aged KO mice.



Online Supplementary Figure S2. Histological examination of the wild-type bone marrow space. (A) Bone marrow section of the femoral shaft of an aged WT mouse stained with Giemsa. Note the absence of trabecular structures. (B) Bone marrow section of the femoral shaft of an aged WT mouse stained for tartrate-resistant acid phosphatase. Magnification: 200x.