Heme oxygenase-1 deficiency alters erythroblastic island formation, steady-state erythropoiesis and red blood cell lifespan in mice

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Supplementary Materials

Mice

Animals used in the current study were backcrossed for ≥14 generations on BALB/c background. Genotypes were verified by genomic PCR using the following primers *Hmox1* 5'-ATGCCCCACTCTACTTCCCTG-3' and 5'-AGGCGGTCTTAGCCTCTTCTG-3' and neo 5'-CTGGGCACAACAGACAATCGG-3' and 5'-AAGCACGAGGAAGCGGTCAG-3'.

Flow cytometric analyses

Blood samples were collected in heparin tubes, and BM cells by flushing femora with phosphate buffered saline (PBS). Bone marrow cells, spleen and liver single cell suspensions were prepared by passing minced tissue pieces through progressively finer Nylon cell strainers (BD Falcon^{TM}). Cells were counted using a handheld automated cell counter (Millipore), blocked on ice for 30 min with FcR Blocking Reagent (Miltenyi Biotec), and then washed once in PBS before incubated with the primary antibodies. A complete list of antibodies for flow cytometry is presented in Supplemental Table S1. Directly conjugated antibodies for flow cytometry were purchased from either eBioscience, BioLegend or Santa Cruz Biotechnology. Erythroblastic island macrophages were characterized as described recently,[1] using propidium iodide (Sigma-Aldrich) and a cocktail of the following antibodies: F4/80 APC, VCAM-1 eFluor450, ER-HRS3 FITC, CD11b Brilliant Violet 711, CD169 PE and Ly6G PE-Cy7. Cells were washed once and resuspended in 1 mL 1% FBS in PBS for FACS analysis. Data was acquired on a LSRII SORP (BD Biosciences) and analyzed on FlowJo software (Tree Star, Inc). Bone marrow cells were gated for propidium iodide-negative single cells prior to gating for F4/80^{+} and VCAM-1^{+} macrophages, then further sub-gated for Ly6G^{+}, CD11b^{+}, CD169^{+} and ER-HR3^{+} antigens.[1] Maturing erythroid cells were assessed by 1 minute incubation of cell pellets with 5 μM of the cell-permeable DNA-binding fluorescent dye DRAQ5 (Alexis Biochemicals).[2] Phosphatidylserine was detected using the

Annexin V AlexaFluor 488 kit (Life Technologies). Stained cells were washed with 1 mL PBS and resuspended in PBS containing 1% FCS and propidium iodide (Sigma), for dead cell exclusion.

EBI isolation and confocal microscopy

BM was obtained from *Hmox*$^{-/-}$, *Hmox1*$^{+/-}$ and *Hmox1*$^{+/+}$ mice by gently flushing the femora using 25-gauge needle and syringe with Iscove's modified Dulbecco medium (IMDM). Aliquots (5 mL) of this suspension were each layered onto 15 mL of IMDM containing 30% fetal bovine serum. After 1 hour, the upper 18 mL were discarded and the lower 2 mL were combined and gently suspended in IMDM containing 1% bovine serum albumin, 100 μg/mL of streptomycin, 100 U/mL penicillin. The cells and cluster cell suspensions were then plated on 15 mm thick glass coverslips in a 12-well tissue culture dish and incubated at 37°C in humidified air with 5% CO_2 to permit cells to attach to the coverslips. After 45 minutes, the medium was removed and the coverslips were rinsed once with PBS to remove all unattached cells.

For confocal microscopy, cells were fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature then rinsed with PBS. Cells were further permeabilized with 0.5% Triton X-100 in PBS for 5 minutes and then rinsed with PBS. After incubating the cells in 2% bovine serum albumin and 0.1% Triton X-100 in PBS for 30 minutes to block non-specific protein binding, fixed cells were treated with AlexaFluor 647-conjugated phalloidin (Life Technologies) diluted in 3% bovine serum albumin/PBS for 45 minutes at room temperature. Cells were then washed three times in PBS and mounted using Vectashield with DAPI. Samples were imaged using a Leica SPEII confocal microscope.

Scanning electron microscopy

Blood was collected via retro-orbital bleed into acid citrate dextrose (ACD), diluted into PBS and centrifuged at 300xg for 10 minutes. Isolated EBI were fixed with 2.5% glutaraldehyde (ProSciTech, Thuringowa, Australia) at room temperature for 2 hours, washed three times with 0.1M sodium cacodylate buffer pH7.4 (ProSciTech) and secondary fixation was carried out using 1% osmium tetroxide (ProSciTech) for 1 hour. EBI were gradually dehydrated using series of ethanol (30% to 100%). The samples were then dried with HMDS for 2 min. Coverslips in the culture dish were placed in a dessicator to air-dry. The coverslips were then carefully mounted onto a suitable aluminum pin stub using carbon tape. The EMITECH K500X Sputter Coater (Quorum Technologies Ltd, United Kingdom) was used to coat the samples with 15 nm layer of gold at 25 mA for 2 minutes. A Hitachi S4500 FEG-SEM equipped with a Colf field-emission source (Australian Centre for Microscopy & Microanalysis (ACMM)) was used for imaging. The coated samples on the pin stubs were examined under 5.0 kV accelerating voltage, with a working distance of 15 – 18 mm. Beam current was in the range of 6 μA to 10 μA. The samples were tilted at an angle of 30° and a Z-height of 10 mm before being imaged. Images were taken digitally using the Esprit EDS software (v1.9, Bruker Corporation, Preston VIC, Australia). False colors and further processing of the images were done on Adobe Photoshop CS3 Extended (v10.0) (Adobe Systems Incorporated, San Jose, California, U.S.A).

For RBC, blood was collected from mice via retro-orbital bleed into ACD, diluted into PBS and centrifuged at 300xg for 10 minutes. The pelleted RBC were then fixed in 2% glutaraldehyde (Electron Microscopy Sciences) in 0.1 M sodium cacodylate overnight at 4°C, followed by two washes of 15 minutes in 0.1 M sodium cacodylate buffer, pH 7.4. Samples were postfixed in 1% osmium tetroxide (ProSciTech) in 0.1 M sodium cacodylate buffer for 1.5 hours at room temperature, followed by two washes of 15 minutes in 0.1 M sodium cacodylate buffer and dehydrated using a graded series of acetone. The samples were critical

point dried using a Leica EM CPD300, mounted on aluminium stubs and sputter-coated with gold prior to imaging on a Zeiss Gemini Ultra-Plus using SmartSEM software (V05.03).

RBC production and lifespan

Briefly, mice were injected via the tail vein with 25 μL of 20 mM carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes) in 75 μL PBS. RBC were quantified by collecting 5 μL blood taken from the tail tip into 45 μL ACD, diluting 10 μL blood/ACD in 990 μL PBS containing 10 μL reference beads (BD Biosciences), and analyzing the number of $CFSE^+$- and $CFSE^-$-RBC by flow cytometry (BD FACScan and FlowJo software). The post-labeling monitoring period involved an initial phase of 5 days where 5 μL of blood were collected daily, followed by a phase where the same volume of blood was collected every 10 days for a total period of 50 days. To assess RBC lifespan in transfusion experiments, labelled RBC from *Hmox1*$^{+/+}$ mice were transfused into *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ recipient mice. To minimize the post-collection handling of RBC, donor mice were injected intravenously with CFSE as described above. Blood was collected into ACD via retro-orbital bleed from the donor mice 18 hours following CFSE injection. The RBC were isolated via centrifugation, washed once in 10 volumes PBS, resuspended in PBS and injected into the recipient mice. The number of transfused labeled RBC in the recipient mice ranged between 6.1 and 13% of total RBC numbers 2 hours after transfusion. Lifespan analysis of the transfused RBC was undertaken as described above. The average red cell lifespan of transfused cells in each mouse was determined assuming their lifespans have a log normal distribution, and fitting a survival curve to the proportion of circulating $CFSE^+$-cells at various times after transfusion, as described previously.[3] Matlab's nonlinear least-squares estimator lsqnonlin was used to perform the data fitting. Estimated lifespans between the groups of mice were compared using a two-way ANOVA followed by a post-hoc Tukey test.

Redox state of peroxiredoxin 2

For native Prx2, freshly collected mouse blood (1 vol) was added to 1 vol of 200 mM *N*-ethylmaleimide in EDTA tubes and incubated at room temperature for 1 hour. For hydrogen peroxide challenge, washed RBC were exposed to different concentrations of H_2O_2 before alkylation with *N*-ethylmaleimide for 1 hour. Protein solutions were added to non-reducing loading buffer in a 1:100 ratio and 5-10 μg proteins then loaded onto 12% SDS-polyacrylamide gels (NuPage, Invitrogen). Following separation under non-reducing condition, proteins were transferred to nitrocellulose membranes (GE Healthcare Bio-Sciences), blocked using 5% milk powder and then incubated with Prx2 antibody (Epitomics) for 1 hour at room temperature. Bands were detected using horseradish-peroxidase conjugated goat anti-rabbit antibody (Dako). Proteins were visualized using chemiluminescence (ECL detection reagent and Hyperfilm ECL, GE Healthcare Bio-Sciences) and analyzed by densitometry using the GelDoc system and QuantityOne software (BioRad).

Biochemical analyses

The Drabkin method was used to determine hemoglobin concentration in blood.[4] Briefly, 20 μL of heparinized whole blood was added to 4 mL of Drabkin reagent (5 mg KCN, 20 mg $K_3Fe(CN)_6$, 100 mg $NaHCO_3$ and 100 μL NP-40 in 100 mL of water) and incubated for 10 minutes at room temperature. Absorbance was then measured at 540 nm and the hemoglobin concentration calculated using $\varepsilon_{540\,nm} = 44\ mol^{-1}\ cm^{-1}$. Plasma hemoglobin was determined by the sensitive two-site enzyme linked immunoassay ELISA (Kamiya Biomedical Company), following the manufacturer's instructions. Erythropoietin (EPO) in mouse plasma was determined using the EPO Quantikine® ELISA (R&D Systems), following the manufacturer's instructions.

Plasma concentrations of heme and bilirubin were determined by LC-tandem mass spectrometry using a 6460 Triple Quadrupole LC/MS (Agilent Technologies), equipped with an ESI source and connected to an Agilent 1200 UHPLC. Plasma samples were diluted 1:10 with ice-cold methanol, placed on ice for 20 minutes and then centrifuged at 16,000xg for 20 minutes. The resulting supernatant was injected onto a RRHD Eclipse Plus C18 column (2.1 x 50 mm, 1.8 μm, Agilent) eluted at 0.3 mL per minute. For heme, gradient elution was used employing Solvent A (5 mM ammonium acetate with 0.1% formic acid) and B (5mM ammonium acetate in methanol with 0.1 % formic acid): 60-85% Solvent B over 10 minutes, 85% Solvent B for 10 minutes, return to 60% Solvent B over 3 minutes and held at 60% Solvent B for 7 minutes. For bilirubin, isocratic elution using 15% Solvent A and 85% Solvent B was used. The mass spectrometer was operated in positive electrospray ion mode and quantification of heme and bilirubin was by multiple reaction monitoring using the largest fragment ion generated by collision-induced dissociation of the $[M+H]^+$ ion was for quantification the following mass spectrometry settings: m/z 616.2→557.2 with fragmentor voltage (FV) = 160 V and collision energy (CE) = 41 V and m/z 585.2→299.2 with FV = 90 V and CE = 17 V, for heme and bilirubin, respectively. Concentrations were calculated from calibration curves generated using authentic standards of hemin (Fluka) and bilirubin chloride (Frontier Scientific). All reagents were of analytical grade.

Statistical analyses

Unless indicated otherwise, statistical analysis was performed using GraphPrism or KaleidaGraph software. Results are expressed as mean value with error bars representing standard error of the mean. The Wilcoxon Rank Sum test or Kruskal–Wallis one-way analysis of variance was used to determine statistical significance unless indicated otherwise. A P-value of <0.05 was considered as statistically significant.

Supplementary References

1. Jacobsen RN, Forristal CE, Raggatt LJ, et al. Mobilization with granulocyte colony-stimulating factor blocks medullar erythropoiesis by depleting $F4/80^{+}VCAM1^{+}CD169^{+}ER\text{-}HR3^{+}Ly6G^{+}$ erythroid island macrophages in the mouse. *Exp Hematol*. 2014;42(7):547-561.
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3. Coupland LA, Cromer D, Davenport MP, Parish CR. A novel fluorescent-based assay reveals that thrombopoietin signaling and Bcl-X(L) influence, respectively, platelet and erythrocyte lifespans. *Exp Hematol*. 2010;38(6):453-461.
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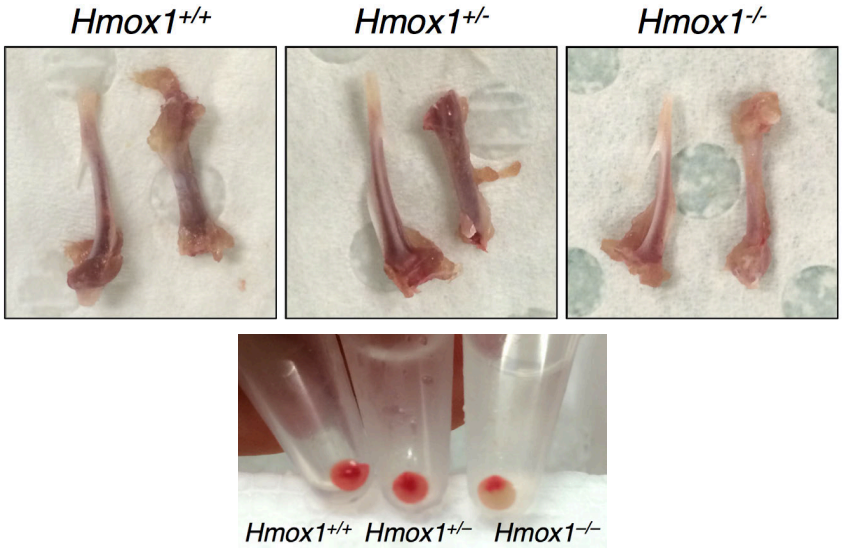
Supplementary Table S1: Antibodies used in this study for flow cytometry

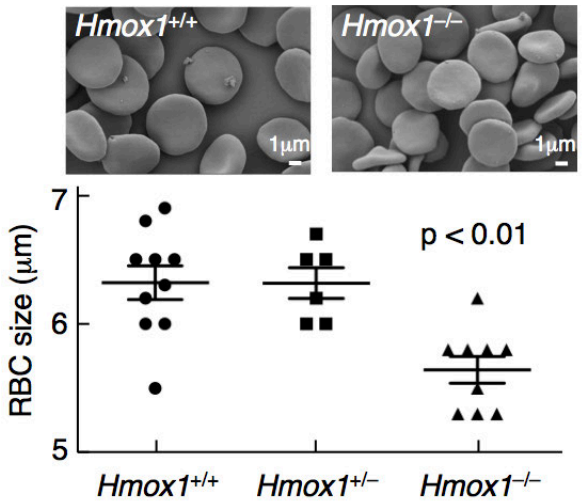
Antibody	Fluorochrome	Clone	Source
CD11b/Mac1	FITC/PE/BV711	M1/70/M1/70	eBioscience/BD Biosciences
CD29/β1-integrin	FITC/PE	HMb1-1	eBioscience
CD45	PE	30-F11	eBioscience
CD49d/α4-integrin	PE	R1-2	Biolegend
CD49e/α5-integrin	PE	MFR5	eBioscience/Biolegend
CD71/Transferrin receptor	FITC/PE	R17217	eBioscience
CD106/VCAM-1	PE/EFLUOR450	429/429	eBioscience
CD169	PE	3D6.112	BioLegend
ER-HRS3	FITC	not specified	Santa Cruz Biotechnology
F4/80	APC	BM8/BM8	BioLegend/eBioscience
Forssman antigen	PE	M1/87	Santa Cruz Biotechnology
Ly6G	PE-Cy7	1A8	BD Biosciences
Ter-119	APC	Ter-119	eBioscience
Tim4	PE	RMT4-54	eBioscience/Biolegend

Supplementary Figure S1. Visual evidence for decrease in total erythroid cell content in BM of *Hmox1*$^{-/-}$ mice.



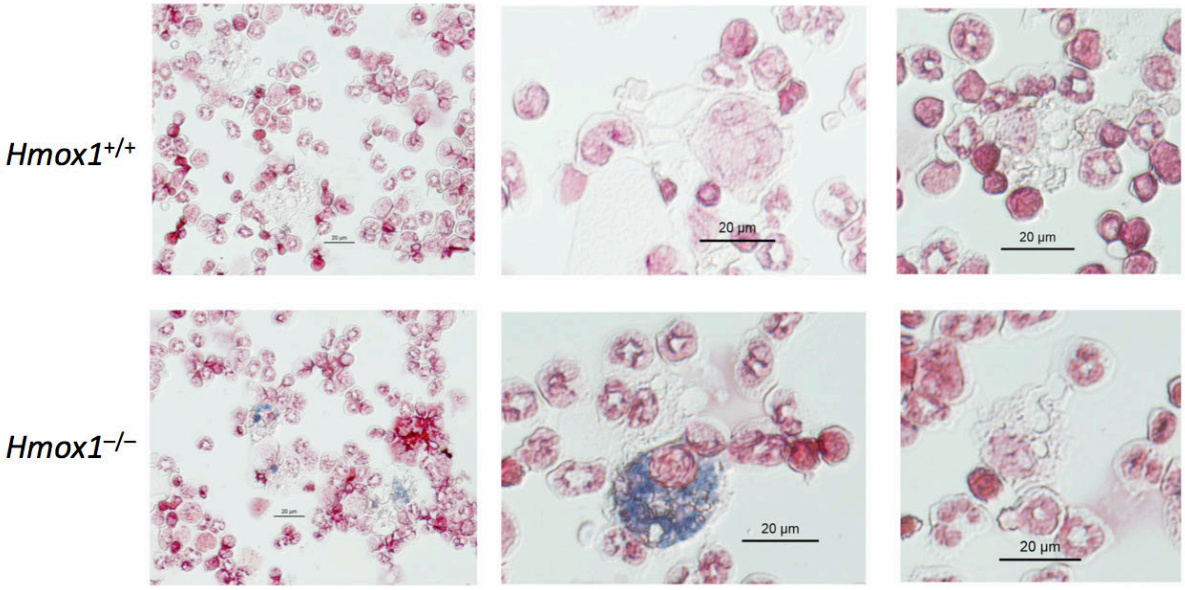
Top, representative images of tibia (left bone) and femur (right bone) dissected from *Hmox1*$^{+/+}$ and *Hmox1*$^{+/-}$ mice showing stronger pigmentation that corresponding bones from *Hmox1*$^{-/-}$ animals, prior to bone marrow isolation. Bottom, bone marrow cells (3 x 10^6) harvested from *Hmox1*$^{+/+}$, *Hmox1*$^{+/-}$ and *Hmox1*$^{-/-}$ mice were centrifuged at 350 g for 5 min. BM cells from *Hmox1*$^{-/-}$ mice typically contained fewer erythroid cells.

Supplementary Figure S2. Decreased RBC size in *Hmox1*$^{-/-}$ compared with *Hmox1*$^{+/+}$ and *Hmox1*$^{+/-}$ mice.



Representative scanning electron micrographs of circulating RBC from *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ mice (top panel). Quantification of the microcytosis arising from *Hmox1* deficiency is shown in bottom panel.

Supplementary Figure S3. Absence of sideroblasts in BM of *Hmox1*$^{-/-}$ mice.



Iron staining of BM cells shows iron in some but not all macrophages from *Hmox1*$^{-/-}$ mice. No ring sideroblasts were observed in bone marrow samples. Iron staining was performed on methanol-fixed cytospun samples using the Iron Staining Kit (Sigma Aldrich) following the manufacturer's instructions. The blue color indicates the presence of iron, whereas the red color is a nuclear counterstain. Images are representative for samples from three independent experiments for each genotype.