

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

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ABSTRACT

Heme oxygenase-1 is critical for iron recycling during red blood cell turnover, whereas its impact on steady-state erythropoiesis and red blood cell lifespan is not known. We show here that in 8- to 14-week old mice, heme oxygenase-1 deficiency adversely affects steady-state erythropoiesis in the bone marrow. This is manifested by a decrease in Ter-119⁺-erythroid cells, abnormal adhesion molecule expression on macrophages and erythroid cells, and a greatly diminished ability to form erythroblastic islands. Compared with wild-type animals, red blood cell size and hemoglobin content are decreased, while the number of circulating red blood cells is increased in heme oxygenase-1 deficient mice, overall leading to microcytic anemia. Heme oxygenase-1 deficiency increases oxidative stress in circulating red blood cells and greatly decreases the frequency of macrophages expressing the phosphatidylserine receptor Tim4 in bone marrow, spleen and liver. Heme oxygenase-1 deficiency increases spleen weight and Ter119⁺-erythroid cells in the spleen, although $\alpha 4\beta 1$ -integrin expression by these cells and splenic macrophages positive for vascular cell adhesion molecule 1 are both decreased. Red blood cell lifespan is prolonged in heme oxygenase-1 deficient mice compared with wild-type mice. Our findings suggest that while macrophages and relevant receptors required for red blood cell formation and removal are substantially depleted in heme oxygenase-1 deficient mice, the extent of anemia in these mice may be ameliorated by the prolonged lifespan of their oxidatively stressed erythrocytes.

Introduction

In healthy adults the constant large-scale production of mature red blood cells (RBC) is counterbalanced by the clearance of aged or damaged RBC. The bone marrow (BM) is the primary erythropoietic organ with the spleen becoming important during acute or chronic stress. Erythroid progenitor cells interact with BM macrophages to form multicellular clusters termed erythroblastic islands (EBI).^{1,2} Within this microenvironment, macrophages are thought to supply the rapidly hemoglobinizing erythroblasts with iron and growth factors. Erythroblasts condense and expel their nuclei in a process termed enucleation.³ BM macrophages engulf and destroy these free nuclei leading to the release of anuclear reticulocytes into the circulation,^{4,5} where they rapidly mature to RBC which then circulate for ~35-50 days in the mouse⁶, and 120 days in the human.

Erythrocyte clearance typically takes place in the spleen, where phagocytes engulf and destroy aged or damaged RBC. Exposure of phosphatidylserine on the RBC surface is a feature of aging, and the recognition of such phosphatidylserine

by Tim4-expressing splenic macrophages leads to RBC engulfment and destruction.^{7,8} A critical stage in RBC clearance is the hemoglobin breakdown and catabolism of released heme into carbon monoxide, iron and biliverdin⁹ by heme oxygenase-1 (encoded by *Hmox1*).¹⁰ Biliverdin reductase then converts biliverdin to the potent antioxidant bilirubin.¹¹

Mice lacking both copies of *Hmox1* exhibit a range of severe defects. Firstly, only ~10-20% of expected *Hmox1*-deficient animals are born alive suggesting that most die *in utero*.¹² Secondly, the *Hmox1*-null mutant animals that reach adulthood show numerous health problems including altered spleen morphology and function, vascular injury, renal and hepatic damage, and an increased sensitivity to oxidative stress and endotoxemia.¹³⁻¹⁵ Moreover, in *Hmox1*^{-/-} mice, initial splenic enlargement progresses to red pulp fibrosis, atrophy, and functional hyposplenism in animals older than 9 months,¹⁶ reminiscent of the asplenia in the extremely rare *HMOX1*-null patients who also present with anemia, microcytosis and abnormal iron metabolism.^{15,17,18} Furthermore, polymorphisms in the *HMOX1* gene promoter which can affect the extent of gene transcription are associated with a

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range of clinical pathologies, including idiopathic recurrent miscarriage,¹⁹ fetal hemoglobin expression in Brazilian patients with sickle cell anemia,²⁰ and pre-eclampsia.²¹

Splenic macrophages are central to whole body iron recycling and return the iron from cleared RBC to the BM for use in erythropoiesis.^{16,22} Hmox1 plays a critical role in this iron recycling and regulates the ability of splenic macrophages to tolerate the toxic heme released during RBC clearance.¹⁶ Hmox1 is expressed in splenic macrophages and is up-regulated in other cell types in response to heme and oxidative stress.²³ Splenic macrophages are significantly decreased in mice lacking Hmox1,¹⁶ resulting in iron redistribution from the spleen and hepatic Kupffer cells to hepatocytes and proximal tubular cells of the kidney.¹⁶ Inappropriate handling of heme and tissue deposition of iron in *Hmox1*^{-/-} mice and *HMOX1*-deficient patients results in increased oxidative stress and vascular cell injury.^{16,17} Hematopoiesis and stress erythropoiesis in *Hmox1*^{+/-} mice has been investigated recently. BM cells from *Hmox1*^{+/-} mice are less capable of reconstituting lethally irradiated recipient animals.²⁴ Moreover, mice with a hematopoietic system reconstituted from *Hmox1*^{+/-} donor animals demonstrate decreased stress erythropoiesis in response to anemia.²⁵

Here, we investigated erythropoiesis in the complete absence of the *Hmox1* gene and protein expression and without exerting exogenous stress in young, 8- to 14-week old mice. We found significant alterations in the BM, circulating and splenic erythroid populations in *Hmox1*^{-/-} mice. In the BM, the number of EBI macrophages was decreased and the expression of adhesion molecules was altered in erythroblast and macrophage populations, manifested as the inability of *Hmox1*-deficient BM to form EBI. *Hmox1*-deficient RBC also showed profound changes in redox biology and lifespan. Splenic erythropoiesis was almost completely absent and splenic macrophages involved in RBC removal were severely depleted. Our findings document defects in RBC production and lifespan resulting from the loss of this crucial heme-catabolizing enzyme.

Methods

Full details of the materials and methods are provided in the *Online Supplementary Methods*.

Mice

Hmox1^{+/-} breeders on a BALB/c background were obtained from Dr MP Soares (Instituto de Gulbenkian de Ciencia, Portugal) from a colony generated originally by Dr SF Yet.²⁶ Male and female mice, 8 to 14 weeks old, *Hmox1*^{-/-}, *Hmox1*^{+/-} and *Hmox1*^{+/+} littermates obtained from *Hmox1*^{+/-} x *Hmox1*^{+/-} breeding, were used for experiments. All procedures were carried out according to the Australian NHMRC guidelines for animal research and were approved by the Animal Care and Ethics Committees of the University of Sydney and the Garvan Institute of Medical Research/St Vincent's Hospital.

Flow cytometry and cytological analysis

Blood, BM, spleen and liver single cell suspensions were treated with FcR-block (MACS Miltenyi Biotech) then with appropriate antibodies for 1 h at 4°C. A list of antibodies is presented in *Online Supplementary Table S1*. The presence of a nucleus or surface membrane phosphatidylserine was assessed as described in the *Online*

Supplementary Methods. Resuspended samples were analyzed using FACSCalibur (Becton Dickinson) and FlowJo software. Iron staining was performed on methanol-fixed cytospun BM samples using the Iron Stain Kit (Sigma Aldrich) according to the manufacturer's instructions.

Isolation and imaging of erythroblastic islands

EBI were obtained from BM as described elsewhere,²⁷ with modifications described in the *Online Supplementary Methods*. For confocal microscopy imaging, cells were permeabilized and fixed, before treatment with AlexaFluor 647-conjugated phalloidin (Life Technologies) and imaging using a Leica SPEII confocal microscope.

Scanning electron microscopy

The pelleted RBC and EBI underwent standard work-up procedures required for scanning electron microscopy imaging, as described in the *Online Supplementary Methods*.

Red blood cell production and lifespan

RBC production and lifespan were determined using *in vivo* carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling,⁶ with slight modifications described in the *Online Supplementary Methods*. The number of CFSE⁻RBC over time indicates RBC production, whereas the number of CFSE⁺-RBC over time indicates lifespan. A second strategy involved transfusing CFSE-labeled RBC from *Hmox1*^{+/+} into *Hmox1*^{+/-} or *Hmox1*^{-/-} mice.

Biochemical analyses

Freshly collected blood (1 vol) was added to 1 vol of 200 mM *N*-ethylmaleimide in EDTA tubes and incubated at room temperature for 1 h, before the native redox state of peroxiredoxin 2 (Prx2) was determined.²⁸ Heparinized blood was centrifuged and cells washed and incubated for 10 min at 37°C with hydrogen peroxide (0–150 μ M). Cells were alkylated with *N*-ethylmaleimide for 1 h. Protein concentrations were determined and Prx2 redox state assessed. Blood hemoglobin was determined by the Drabkin method,²⁹ while plasma hemoglobin and erythropoietin were assessed by enzyme-linked immunosorbent assay. Heme and bilirubin were analyzed by liquid chromatography-tandem mass spectrometry.

Statistical analysis

Details regarding the statistical analysis are presented in the *Online Supplementary Methods*.

Results

By 20 weeks of age, 129/Sv x C57BL/6 chimeras of *Hmox1*^{-/-} mice display systemic inflammation and disordered iron metabolism characterized by microcytic anemia, anisocytosis, decreased serum iron, splenomegaly, and iron deposition in the liver and kidney.^{12,16} As genetic background and age affect phenotypes, the current study used comparatively young (8 to 14 weeks old) *Hmox1*^{-/-} mice back-crossed for ≥ 14 generations onto the BALB/c genetic background. Compared with *Hmox1*^{+/+} littermates, *Hmox1*^{-/-} mice also showed splenomegaly and a decrease in hematocrit and hemoglobin (Table 1), indicating that *Hmox1* deficiency causes anemia even in young adult mice prior to the progressive inflammation present in older animals.¹² Hematologic parameters of *Hmox1*^{+/-} animals have not been reported previously even though a decrease in *Hmox1* is associated with vascular and hematopoietic

alterations. Most parameters were not altered in *Hmox1*^{+/-} mice compared with wild-type animals, except for the significant increase in plasma hemoglobin in heterozygous mice. This demonstrates that *Hmox1* gene dosage is important in regulating hemoglobin clearance, although this was not investigated further. We also determined plasma concentrations of heme and bilirubin, the substrate for *Hmox1* and end-product of heme catabolism, respectively, as such information is currently lacking. We observed that plasma hemoglobin and heme were increased and bilirubin decreased in *Hmox1*^{+/-} mice compared with *Hmox1*^{+/+} and *Hmox1*^{-/-} mice (Table 1).

Hmox1 deficiency alters steady-state erythropoiesis

As the BM is the primary site for erythropoiesis, we first examined the frequency of BM erythroid cells identified by the lineage-specific surface antigen Ter-119. The proportion of nucleated and enucleated BM cells being Ter-119⁺ was decreased significantly in *Hmox1*^{+/-} compared with *Hmox1*^{+/+} and *Hmox1*^{-/-} mice (Figure 1A). Consistent with the suggested impairment of steady-state erythropoiesis in *Hmox1* deficiency, bones and BM cells collected from *Hmox1*^{+/+} and *Hmox1*^{-/-} mice generally appeared more pigmented than their counterparts from *Hmox1*^{+/-} mice (Online Supplementary Figure S1). We next subjected Ter-119⁺CD71⁺ BM cells to flow cytometry to quantify erythroid cells as they differentiate from pro-erythroblasts (R1), into basophilic (R2), polychromatophilic (R3) and orthochromatophilic erythroblasts (R4) (Figure 1B), as verified previously.³⁰ *Hmox1* deficiency caused a blockade of erythroid maturation in the R1 stage, leading to a significant decrease in R3 (Figure 1B). Similar results were obtained when the different erythroid developmental stages were segregated using expression of Ter-119, the adhesion molecule CD44 and cell size³¹ (Figure 1C). A consistent reduction in the frequency of "population V" cells was observed. Both R3 and "population V" identify reticulocytes.

The above results show that *Hmox1* regulates steady-state erythropoiesis, and they suggest that *Hmox1* deficiency decreases the frequency of reticulocytes in the BM. We therefore asked if *Hmox1* deficiency causes a decrease in the numbers of RBC formed and released into the circulation. To do so, we determined the time-dependent appearance of CFSE-RBC in the circulation of mice after *in vivo* labeling of their blood cells with CFSE.³² The numbers of RBC released into circulation were comparable in *Hmox1*^{-/-}, *Hmox1*^{+/-} and *Hmox1*^{+/+} mice (Figure 1D). However, *Hmox1* deficiency caused a significant decrease in the mean corpuscular diameter, volume and hemoglobin content of circulating RBC (Table 1), as has been reported previously for older animals.¹² The extent of decrease in RBC corpuscular hemoglobin was comparable to the 20% decrease in whole blood hemoglobin, whereas the ratio of RBC corpuscular hemoglobin to volume was unaffected by *Hmox1* deficiency (Table 1). Scanning electron microscopy and size assessment (Online Supplementary Figure S2) confirmed these findings, indicating that the abnormal erythropoiesis observed in *Hmox1* deficiency results in microcytic anemia and an associated decrease in whole blood hemoglobin.

Hmox1 deficiency disrupts bone marrow macrophage-erythroblast interactions

During erythroid maturation, erythroid progenitors

Table 1. Characteristics of mice with *Hmox1* deficiency.

| Parameter | <i>Hmox1</i> ^{+/+} | <i>Hmox1</i> ^{+/-} | <i>Hmox1</i> ^{-/-} |
|-------------------------------------|-----------------------------|-----------------------------|-----------------------------|
| Spleen weight (mg) | 112 ± 4 (n=11) | 113 ± 7 (n=5) | 479 ± 27* (n=8) |
| Hematocrit (%) | 47.0 ± 0.7 (n=15) | 47.0 ± 1.7 (n=6) | 39.3 ± 1.6* (n=11) |
| Whole blood hemoglobin (g/dL) | 16.1 ± 0.3 (n=11) | 16.4 ± 0.3 (n=8) | 12.9 ± 0.2* (n=6) |
| RBC numbers (x 10 ¹² /L) | 11.4 ± 0.3 (n=6) | 11.9 ± 1.0 (n=3) | 13.6 ± 1.5* (n=4) |
| Red blood cell diameter (μm) | 6.3 ± 0.1 (n=10) | 6.3 ± 0.1 (n=6) | 5.6 ± 0.1* (n=9) |
| Mean corpuscular volume, (fL) | 39 ± 1 (n=8) | 41.9 ± 1.8 (n=6) | 32.4 ± 1.7* (n=8) |
| Mean corpuscular hemoglobin, (pg) | 16.2 ± 1 (n=8) | 13.6 ± 1 (n=6) | 11.2 ± 0.9* (n=8) |
| MCH/MCV (pg/fL) | 0.42 ± 0.03 (n=8) | 0.33 ± 0.04 (n=6) | 0.36 ± 0.04 (n=8) |
| Plasma erythropoietin (pg/mL) | 103 ± 8 (n=4) | 100 ± 7 (n=5) | 148 ± 14* (n=5) |
| Plasma hemoglobin (μg/mL) | 78 ± 3 (n=3) | 227 ± 8* (n=4) | 579 ± 258* (n=6) |
| Plasma heme (μmol/L) | 0.5 ± 0.04 (n=3) | 0.9 ± 0.3 (n=3) | 2.2 ± 0.5* (n=5) |
| Plasma bilirubin (μmol/L) | 1.9 ± 0.6 (n=3) | 2.2 ± 0.3 (n=3) | 0.7 ± 0.1* (n=5) |

Individual parameters were determined as described in the Online Supplementary Methods. Results shown represent mean ± SEM. *P<0.05 compared with *Hmox1*^{+/+}. RBC: red blood cell; MCH: mean corpuscular hemoglobin; MCV: mean corpuscular volume.

interact with the central macrophages of EBI in BM. We first determined *Hmox1* expression in EBI by immunostaining and confocal imaging. As can be seen (Figure 2A), *Hmox1* protein is abundantly expressed by the central EBI macrophages. We next assessed the impact of *Hmox1* deficiency on EBI. EBI from wild-type mice yielded typical 'EBI rosettes', consisting of F4/80⁺ macrophages and their companion Ter-119⁺ erythroblasts (Figure 2B, middle panel). Despite repeated attempts, clusters from *Hmox1*^{+/-} BM did not remain adhered to the coverslip after the numerous incubation and wash steps involved in the immunolabeling procedure. Rare individual macrophages could be observed (Figure 2B, right panel), but these were never observed in clusters associating with erythroblasts. To examine the impact of *Hmox1* on EBI formation further, EBI were immediately fixed and processed for scanning electron microscopy. These experiments showed that wild-type BM macrophages readily adhered to and spread across the surface of the coverslip with adhering erythroid progenitors and, occasionally, reticulocytes (Figure 2C). The morphology of most *Hmox1*^{+/-} EBI was similar to that of wild-type EBI, although some appeared disrupted and their macrophages ruffled. Compared with wild-type EBI, the few EBI that could be obtained from *Hmox1*^{+/-} mice were smaller, contained fewer erythroblasts and showed profound abnormalities in structure and morphology: macrophages appeared dome-shaped (rather than flattened) and stressed, with a ruffled and abnormal cell membrane (Figure 2C, bottom right).

To identify the causes of EBI failure in *Hmox1*^{+/-} mice, we

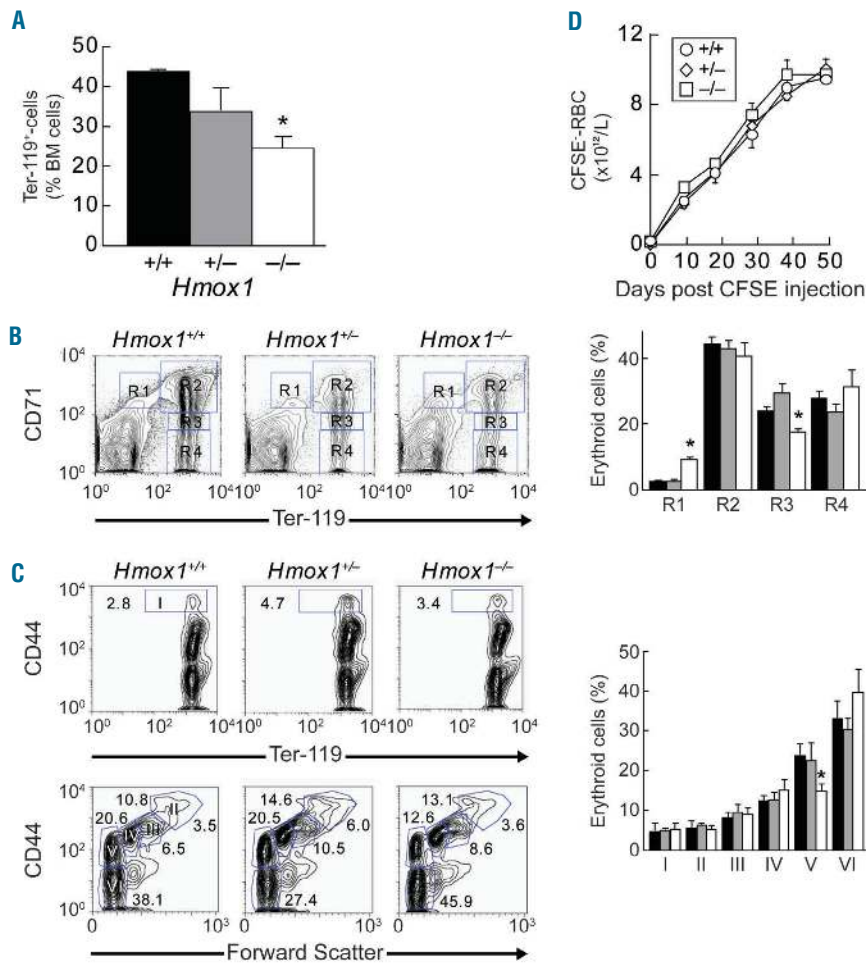


Figure 1. Altered steady-state erythropoiesis in the BM of *Hmox1*^{-/-} mice. Erythroid maturation in BM of *Hmox1*^{+/+} (n=6), *Hmox1*^{+/-} (n=3) and *Hmox1*^{-/-} mice (n=5) was assessed by flow cytometry. (A) Proportion of nucleated and enucleated Ter-119⁺ BM cells. (B) Representative flow cytometric profiles and quantification of maturing erythroid populations R1-R4 as assessed by Ter-119 and CD71 surface expression. Results show the different populations as a percent of total cells. (C) Representative flow cytometric profiles and quantification of stages of erythroid differentiation as assessed by Ter-119 and CD44 expression in combination with forward scatter.³¹ (D) Graphic representation of the number of CFSE^{negative}-RBC over 48 days in *Hmox1*^{+/+} (circles), *Hmox1*^{+/-} (diamonds) and *Hmox1*^{-/-} mice (squares) (n=3 each). *P<0.05 compared with *Hmox1*^{+/+} and *Hmox1*^{+/-}.

examined the expression of $\alpha 4\beta 1$ -integrin on BM Ter-119⁺-erythroid cells, as erythroid cells express *Hmox1*.³³ $\alpha 4$ -integrin regulates terminal erythroid maturation,³⁴ and $\alpha 4\beta 1$ -integrin interaction with macrophage vascular cell adhesion molecule 1 (VCAM-1) is a key determinant of EBI formation.^{1,35} Ter-119⁺-cells of *Hmox1*^{-/-} mice expressed higher levels of $\alpha 4\beta 1$ -integrin than the corresponding cells from *Hmox1*^{+/+} and *Hmox1*^{+/-} animals (Figure 3A), indicating that limited erythroid integrin expression was not responsible for the observed failure of BM from *Hmox1*^{-/-} mice to form EBI.

We next employed a recently described detailed characterization of EBI macrophages, using a cocktail of differently labeled antibodies directed against six antigens.³⁶ BM from *Hmox1*^{-/-} mice had significantly decreased numbers of EBI macrophages expressing the surface antigens F4/80, VCAM 1, CD11b, Ly6G, CD169 and ER-HR3 (Figure 3B). Consistent with this observation, the expression of the glycosphingolipid Forssman antigen, a specific marker for EBI-macrophages,³⁷ was decreased profoundly in BM macrophages of *Hmox1*^{-/-} compared with *Hmox1*^{+/+} and *Hmox1*^{+/-} mice (Figure 3C). These results indicate that BM of *Hmox1*^{-/-} animals have a limited ability to form EBI because the numbers of EBI macrophages are decreased. Iron staining of BM cells revealed a build-up of redox-

active iron in macrophages or *Hmox1*^{-/-} compared with *Hmox1*^{+/+} mice (Online Supplementary Figure S3). Interestingly, despite the defects in iron metabolism in *Hmox1*^{-/-} mice, no ring sideroblasts were observed.

Hmox1 deficiency alters circulating and splenic erythroid population frequency

Plasma erythropoietin was significantly increased in *Hmox1*^{-/-} compared with *Hmox1*^{+/-} and *Hmox1*^{+/+} mice (Table 1). Correspondingly, we observed an increase in circulating nucleated erythroid (Figure 4A) and $\alpha 4$ -integrin⁺Ter-119⁺ cells (Figure 4B). The latter include both erythroblasts and reticulocytes. Compared with *Hmox1*^{+/+}, circulating Ter-119⁺CD71⁺-cells were increased in *Hmox1*^{-/-} mice (Figure 4C), suggesting that erythroblastosis takes place in these animals.

We next examined whether the observed erythroblastosis was associated with increased erythropoiesis in the spleen. The proportion of splenic Ter-119⁺-cells was increased significantly in *Hmox1*^{-/-} mice (Figure 4D). This increase, combined with the decrease in Ter-119⁺ cells in the BM (Figure 1A), suggest that the erythropoiesis defect seen in *Hmox1*^{-/-} mice is specific for BM. However, the spleen of *Hmox1*^{-/-} mice also exhibited decreased basophilic (R2) and polychromatophilic (R3) erythroblast

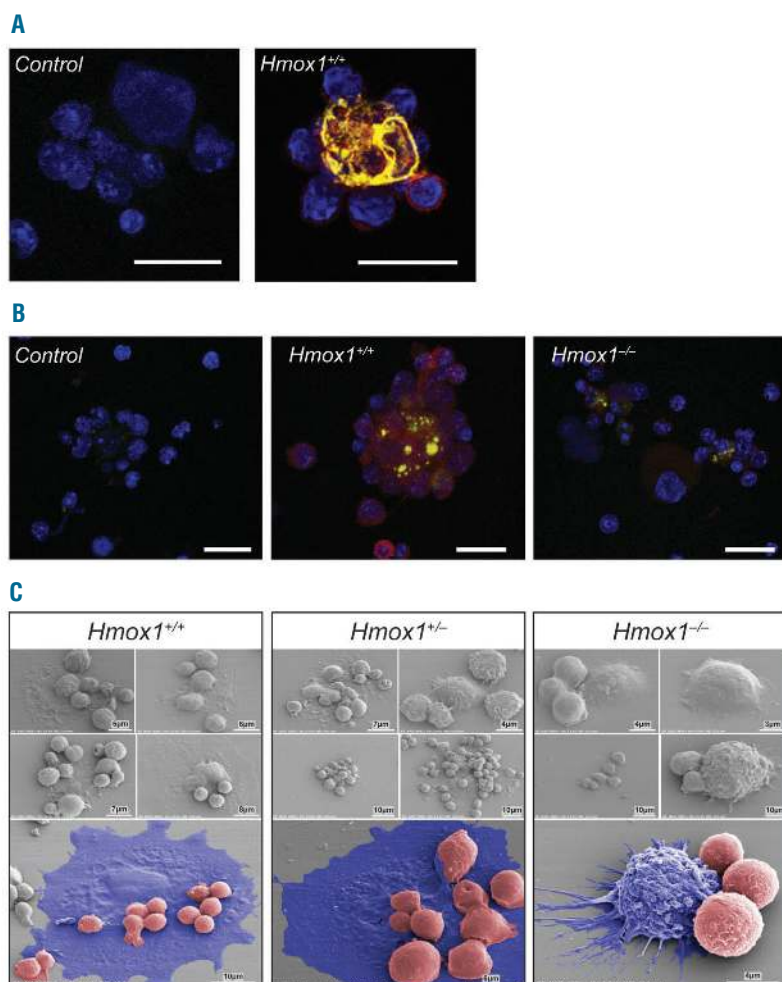


Figure 2. EBI formation is defective in *Hmox1*^{-/-} mice. (A) *Hmox1* is expressed by EBI central macrophages. EBI were obtained as described in the *Methods*, fixed and stained with isotype control antibody (left panel) or antibodies against *Hmox1* (yellow) and F4/80 (red). Cells were counterstained with DAPI to indicate the nuclei (blue). *Hmox1* is abundantly expressed by F4/80⁺ EBI central macrophages (right panel). (B) Macrophages from BM of *Hmox1*^{-/-} mice cannot be identified in multicellular clusters. EBI assays were performed as described in the *Methods* from wild-type and *Hmox1*-deficient bone marrow, fixed and immunostained with Ter-119 (green) and F4/80 (red) antibodies. Multicellular EBI could be identified readily in wild-type samples (middle panel) whereas in samples from *Hmox1*^{-/-} mice BM adherent macrophages could be detected as individual cells. (C) EBI morphology revealed by scanning electron microscopy. EBI, obtained from BM of *Hmox1*^{+/+}, *Hmox1*^{+/-} and *Hmox1*^{-/-} mice as described in the *Methods*, were assessed by scanning electron microscopy. Five EBI are shown for each genotype. Bottom panels show pseudo-colored images with macrophages in blue and hematopoietic progenitor cells in red. *Hmox1*^{+/+} EBI (left panel) attached to and spread across the surface of the glass coverslip with smaller erythroblasts clearly adherent. *Hmox1*^{+/-} EBI (middle panel) showed different morphology, ranging from those resembling wild-type EBI (top left, middle and bottom panels) to EBI showing abnormal macrophages (top right). *Hmox1*^{-/-} EBI (right panel) were repeatedly observed with abnormal morphology. *Hmox1*^{-/-} macrophages failed to extend, spread out and tether hematopoietic progenitor cells. *Hmox1*^{-/-} macrophages failed to spread across the coverslip and in some cases showed signs of stress with ruffled cell membranes and erratic cytoplasmic projections (bottom pseudo-colored panel).

frequency, while the most mature erythroid cells (R4) were increased (Figure 4E), suggesting that stress erythropoiesis was also affected by *Hmox1* deficiency. To assess this further, we examined spleen Ter-119⁺-cells for their expression of α 4-integrin as well as α 5-integrin. The latter regulates the interaction of stress erythroblasts with the splenic microenvironment.³⁸ The expression of both α 4- (Figure 4F) and α 5-integrin (*data not shown*) in splenic erythroid cells of *Hmox1*^{-/-} mice was decreased substantially compared with the expression in *Hmox1*^{+/+} or *Hmox1*^{+/-} animals. This suggests that stress erythropoiesis, characterized by the influence of large numbers of integrin-expressing R2 and R3 erythroblasts, is not a major player in the maintenance of relatively normal RBC levels in *Hmox1*^{-/-} mice.

***Hmox1* deficiency increases red blood cell lifespan**

We next examined the impact of *Hmox1* deficiency on parameters known to regulate the removal of circulating RBC. For this, RBC were assessed first for signs of increased oxidative stress, using oxidized Prx2 as a marker. Prx2 is a thiol-dependent peroxidase essential for sustaining the lifespan of RBC in mice,³⁹ and the third most abun-

dant RBC protein.⁴⁰ Upon reaction with hydrogen peroxide (H₂O₂), the Prx2 monomer is oxidized to two forms of dimers that contain one or two disulfide bonds and that can be separated and detected by western blotting.⁴¹ An increase in the ratio of Prx2 dimers to monomers reflects increased oxidative stress.^{40,41} When treated *in vitro* with reagent H₂O₂, Prx2 dimers formed at lower oxidant concentrations in isolated RBC from *Hmox1*^{-/-} compared with *Hmox1*^{+/+} mice (Figure 5A), consistent with a previous study¹⁴ showing that RBC from *Hmox1*^{-/-} mice were hypersensitive to oxidants *ex vivo*. In addition, freshly isolated RBC from *Hmox1*^{-/-} mice contained a higher proportion of Prx2 as dimers compared with cells from *Hmox1*^{+/+} mice even in the absence of an added oxidant (Figure 5B). This shows for the first time that *Hmox1* deficiency increases endogenous oxidative stress in circulating RBC, possibly as a consequence of the elevated plasma concentrations of the pro-oxidant heme (Table 1).

The rate of removal of circulating RBC was then measured by *in vivo* labeling of cells with CFSE.³² Compared with RBC in *Hmox1*^{+/-} and *Hmox1*^{+/+} mice, RBC in *Hmox1*^{-/-} mice were removed at a significantly slower rate, as determined by the percentage of CFSE⁺-RBC remaining over

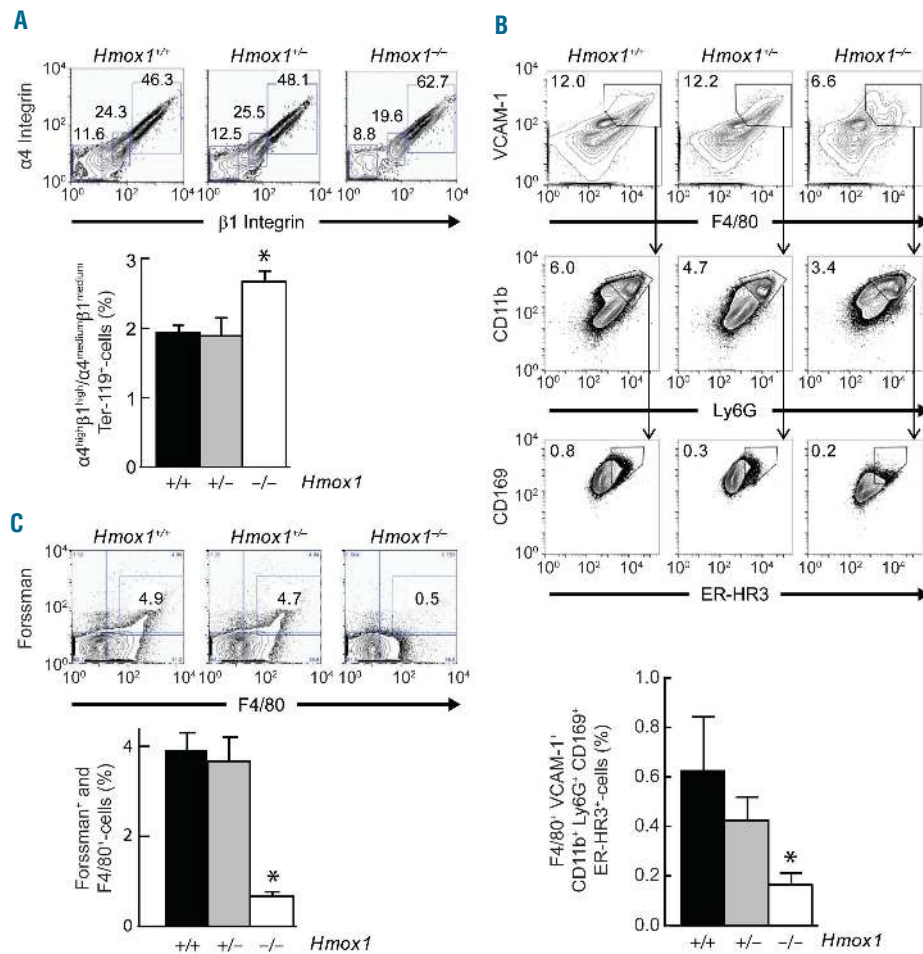


Figure 3. *Hmox1* deficiency reduces the frequency of EBI island macrophages. (A) Representative flow cytometric profiles (top) and quantification (bottom) of $\alpha 4 \beta 1$ integrin expression on Ter-119⁺ cells from BM of *Hmox1*^{+/+} (n = 8), *Hmox1*^{+/-} (n = 4) and *Hmox1*^{-/-} mice (n = 6). (B) Representative flow cytometric profiles (top) and quantification (bottom) of macrophages expressing F4/80⁺ VCAM-1⁺ CD11b⁺ Ly6G⁻ CD169⁺ ER-HR3⁺, showing decreased EBI macrophages in the BM of *Hmox1*^{-/-} (n = 3) compared to *Hmox1*^{+/+} (n = 3) and *Hmox1*^{+/-} (n = 3) mice. (C) Representative flow cytometric profiles (top) and quantification (bottom) indicate that BM F4/80⁺ macrophages of *Hmox1*^{-/-} mice (n = 8) have decreased expression of the Forssman antigen, specific for EBI central macrophages, compared with *Hmox1*^{+/+} (n = 10) and *Hmox1*^{+/-} (n = 5) mice. **P* < 0.05 compared with *Hmox1*^{+/+} and *Hmox1*^{+/-}.

time (Figure 5C). The estimated average lifespan for RBC in *Hmox1*^{-/-} mice was longer (48.8 ± 0.1 days) than that for *Hmox1*^{+/+} and *Hmox1*^{+/-} mice (i.e., 44.1 ± 0.8 and 44.0 ± 2.5 days, respectively; mean ± SEM, n = 3 for each of the three genotypes). These results suggest that the removal of senescent RBC was compromised in *Hmox1*^{-/-} mice. We therefore performed transfer lifespan studies with CFSE-labeled RBC. We observed that RBC from *Hmox1*^{+/+} mice were removed significantly slower in *Hmox1*^{-/-} recipients than in *Hmox1*^{+/+} recipients (Figure 5D), consistent with the notion that RBC lifespan is increased in *Hmox1*^{-/-} mice as a result of extrinsic factors, i.e., compromised RBC removal, rather than RBC intrinsic factors. Consistent with this, splenic F4/80⁺ macrophages expressing CD11b (Figure 5E) and VCAM-1 (Figure 5F) were severely depleted in *Hmox1*^{-/-} mice, and we found no differences in the phosphatidylserine content or osmotic fragility of blood RBC from *Hmox1*^{+/+} and *Hmox1*^{-/-} mice (data not shown).

The liver can serve as an extramedullary hematopoietic organ and also clear aged RBC under stress conditions. However, CD71⁺Ter-119⁺ erythroblasts occurred with comparable frequency in dispersed liver cells of *Hmox1*^{+/+}, *Hmox1*^{+/-} and *Hmox1*^{-/-} mice (Figure 6A). Hepatic F4/80⁺ cells were decreased in *Hmox1*^{-/-} mice (data not shown), although not as drastically as observed in the spleen of

these animals. Similarly, macrophages lacking VCAM-1 and the phosphatidylserine receptor Tim4 were more abundant in the liver of *Hmox1*^{-/-} than *Hmox1*^{+/+} and *Hmox1*^{+/-} mice (Figure 6B), with this decrease in Tim4-expressing F4/80⁺ cells being less severe in the liver than in BM and spleen (Figure 6C). These results indicate that the liver in animals lacking *Hmox1* does not engage consistently in erythropoiesis, and that the population of hepatic macrophages responsible for RBC clearance is decreased in *Hmox1* deficiency.

Discussion

The beginning and end of an erythrocyte's life are book-ended by interactions with macrophages: BM macrophages are considered essential supportive elements in RBC formation and maturation,^{42,43} and splenic macrophages remove aged or damaged RBC. *Hmox1* plays a critical role in the recycling of whole body iron by releasing iron from heme derived from RBC being removed by splenic macrophages; such iron is then made available for the next wave of emerging erythroid cells.¹⁶ Free heme is a strong oxidant and cytotoxic,⁴⁴ and the inability of *Hmox1*-deficient splenic macrophages to toler-

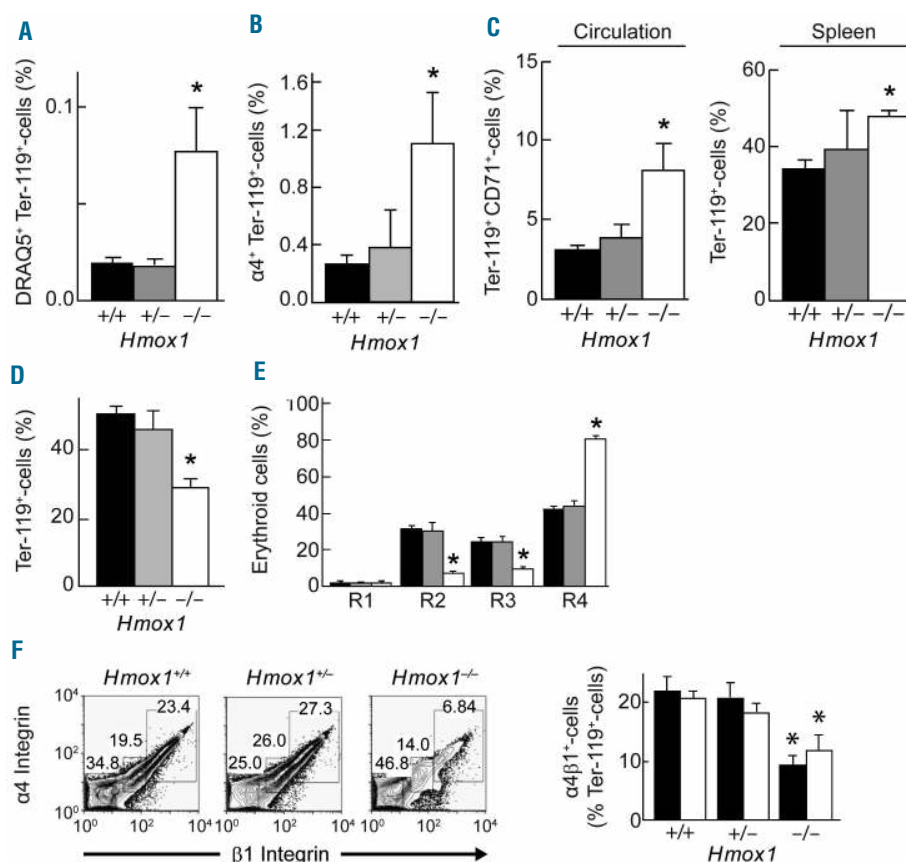


Figure 4. Absence of *Hmx1* alters circulating and splenic erythroid population frequency. *Hmx1* deficiency increases circulating erythroid cells that stain with (A) DRAQ5, (B) the erythroblast marker $\alpha 4$ -integrin, and (C) CD71 (n = 5, 3 and 6 for *Hmx1*^{+/+}, *Hmx1*^{+/-} and *Hmx1*^{-/-} mice, respectively). (D) Increased frequency in Ter-119⁺ erythroid cells in the spleen of *Hmx1*^{-/-} (n = 5) compared with *Hmx1*^{+/+} (n = 6) and *Hmx1*^{+/-} (n = 3) mice. (E) Splenic erythroid maturation in *Hmx1*^{+/+} (black, n = 6), *Hmx1*^{+/-} (gray, n = 3) and *Hmx1*^{-/-} mice (white, n = 5) as assessed by expression of Ter-119⁺ and CD71⁺. (F) Decreased frequency of Ter-119⁺-erythroid cells expressing $\alpha 4\beta 1$ -integrin (left panel), $\alpha 4\beta 1^{\text{high}}$ (black) or $\alpha 4\beta 1^{\text{med}}$ integrin (white) (right panel). Data show representative flow cytometric profiles and their quantification (n = 3-10 for different groups). *P < 0.05 compared with *Hmx1*^{+/+} and *Hmx1*^{+/-} mice.

ate heme leads to their death and disruption of the iron recycling process.¹² Here, we show that *Hmx1* also plays significant roles in regulating erythroid cell-macrophage interactions in the BM.

BM from mice lacking *Hmx1* is severely depleted of EBI macrophages. This is associated with a decrease in the expression of VCAM-1 (Figure 3B, top panels), necessary for interactions with $\alpha 4\beta 1$ integrin on neighboring erythroblasts. During erythroid cell maturation, the nucleus becomes condensed and expelled, surrounded by a thin layer of hemoglobin-containing cytoplasm⁴⁵ and encapsulated in an erythroid cell membrane replete with $\alpha 4\beta 1$ integrin.⁴⁶ We propose that these hemoglobin-containing, expelled nuclei are engulfed preferentially by VCAM-1⁺-macrophages, leading to *Hmx1* induction seen in wild-type EBI macrophages (Figure 2A). We further propose that an inability to catabolize the heme present in 'pyrenocytes'⁴⁷ leads to the selective death of VCAM-1⁺-macrophages in *Hmx1* deficiency. Such proposed selective loss of VCAM-1⁺-macrophages, rather than a decrease in VCAM-1 expression by BM macrophages, is also supported by the observation that *Hmx1* deficiency increases⁴⁷ and *Hmx1* induction decreases VCAM-1 expression.⁴⁷⁻⁴⁹ These changes in erythroblast-macrophage interactions are exemplified best by the scanning electron microscopy analysis of EBI (Figure 2C). High-resolution images of EBI illustrate the morphology of the central island macrophage and erythroblasts attached to it. In BM from *Hmx1*^{-/-} mice, we observed highly abnormal EBI

macrophages with domed morphology rather than flattened, spread-out morphology. These EBI also lacked clear interactions with erythroblasts. Thus, normal EBI formation no longer appears possible in *Hmx1* deficiency. This deficiency likely contributes to alterations in erythropoiesis including microcytosis, abnormal cell membranes, reticulocytosis and erythroblastosis.

Few individuals are born with complete *HMOX1* deficiency. These rare patients exhibit numerous similarities to the mouse null mutant. Notably, erythropoiesis does occur although it is abnormal. *HMOX1*-deficient patients also show altered monocyte phenotype and functions including phagocytosis.¹⁵ BM macrophage phenotype and functions have not been assessed in *HMOX1*-deficient patients. The erythroid defects observed in *HMOX1*-deficient patients could, therefore, also arise from defective erythroid cell-macrophage interactions in the BM, as we have observed in the null mutant mouse model. Furthermore, decreased expression of *HMOX1* due to polymorphisms in the *HMOX1* gene promoter is associated with a number of pathological conditions including difficulties in pregnancy. It is feasible that the differences observed in the *Hmx1*^{+/-} animals (increased plasma hemoglobin for example) are involved in the pathologies observed in patients with *HMOX1* polymorphisms.

Our studies provide direct evidence that *Hmx1* deficiency increases RBC lifespan, likely by attenuating macrophage-mediated RBC removal in the spleen, liver and BM by decreasing both macrophage numbers and

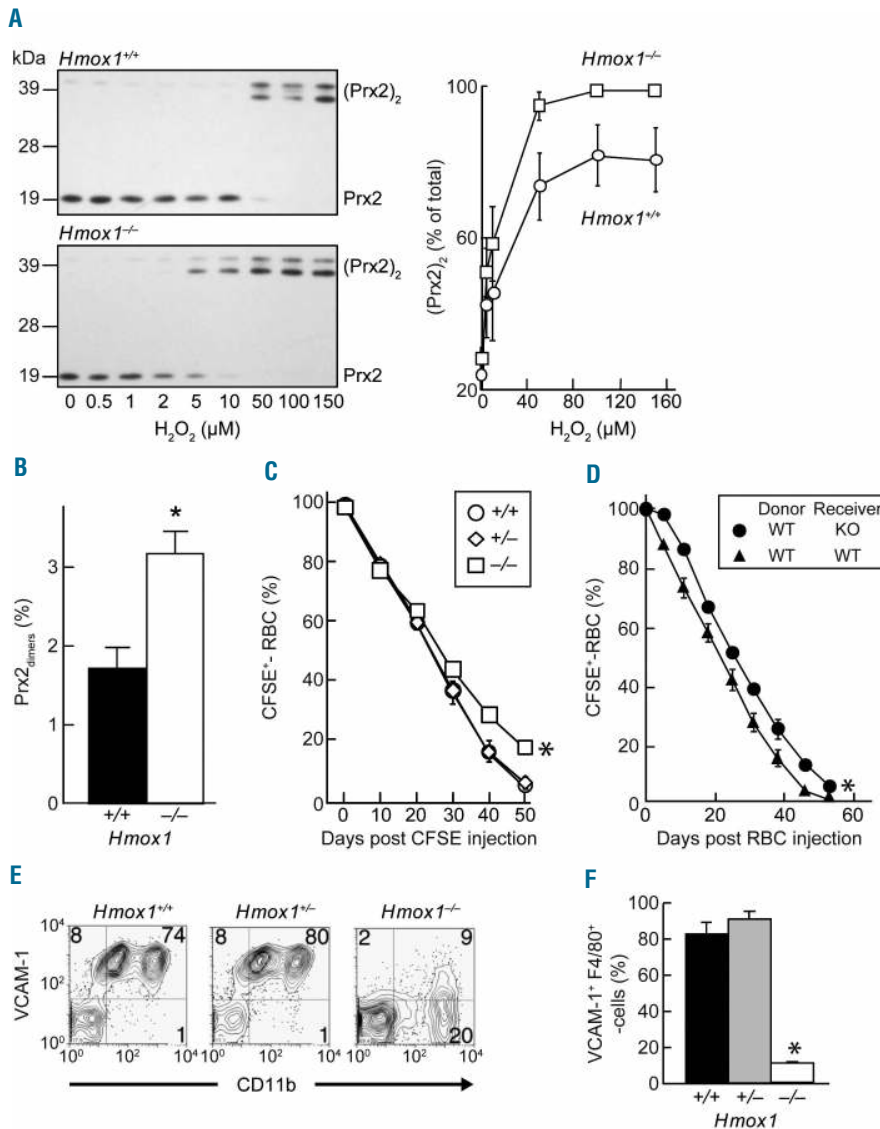


Figure 5. Hmox1 deficiency increases systemic oxidative stress and RBC lifespan. (A) Greater susceptibility of RBC from *Hmox1*^{-/-} (n = 6) than *Hmox1*^{+/+} animals (n = 7) to oxidation induced by added H₂O₂. Representative western blots (left) showing oxidation of Prx2 monomer to Prx2 dimers with increasing concentrations of H₂O₂, with the upper and lower dimer band representing Prx2 with one and two disulfide bonds, respectively.⁴¹ Graphic representation of the densitometry data on the right. (B) Increased systemic endogenous oxidative stress in *Hmox1* deficiency indicated by an increase in the percentage of endogenous RBC Prx2 present as dimers in *Hmox1*^{-/-} (n = 6) compared with wild-type mice (n = 7). (C) Increase in the lifespan of RBC in *Hmox1*^{-/-} (squares) compared with *Hmox1*^{+/+} (circles) and *Hmox1*^{+/-} mice (diamonds) (n = 3 each), as determined by the percentage of CFSE⁺ RBC detected over a 48-day period following *in vivo* CFSE labeling. *P < 0.05 compared with *Hmox1*^{+/+} and *Hmox1*^{+/-} as assessed by two-way ANOVA. (D) Increased lifespan of *in vivo* CFSE-labeled RBC from wild-type (WT) after transfer to *Hmox1*^{-/-} compared with *Hmox1*^{+/+} mice, determined (n = 3 per treatment). *P < 0.05 compared with *Hmox1*^{+/+} and *Hmox1*^{+/-} as assessed by two-way ANOVA. (E, F) Decreased frequency of F4/80⁺ macrophages expressing CD11b and VCAM-1 in *Hmox1*^{-/-} compared with *Hmox1*^{+/+} and *Hmox1*^{+/-} mice (n = 3 each). *P < 0.05 compared with *Hmox1*^{+/+} and *Hmox1*^{+/-}.

Tim4 expression. This may be explained in ways analogous to the specific depletion of BM VCAM-1⁺ macrophages (see above). Whether the elevated oxidative stress experienced by RBC from *Hmox1*^{-/-} mice and indicated by their increased content of oxidized Prx2²⁸ affects lifespan remains to be investigated, e.g., by transfer experiments of labeled RBC from *Hmox1*^{+/+} mice into *Hmox1*^{+/+} and *Hmox1*^{-/-} mice. The corresponding decrease in non-oxidized Prx2 could conceivably attenuate RBC lifespan and increase compensatory erythropoietin synthesis,³⁹ as seen in *Hmox1*^{-/-} mice. Because Hmox1 is not present in RBC, we interpret their increased content of oxidized Prx2 in *Hmox1* deficiency as the result of an increased exposure to oxidants rather than a decrease in antioxidant defenses.¹³ This interpretation is consistent with our observation that Hmox1 deficiency is associated with elevated plasma concentrations of the pro-oxidant heme (Table 1).

The present study does not reveal precisely how RBC are removed in Hmox1 deficiency. Previous studies have

reported the accumulation of iron in hepatocytes and kidneys of *Hmox1*^{-/-} mice,^{12,16} suggesting that cells in these tissues may compensate for decreased RBC clearance by splenic macrophages and the associated reduced heme degradation. What is clear from these previous studies,^{12,16} however, is that such (or any other) compensatory heme catabolism operative in Hmox1 deficiency is less efficient in iron recycling compared with Hmox1-mediated heme degradation, as *Hmox1*^{-/-} mice are anemic. Our studies argue against a fully compensatory role for the constitutively expressed heme oxygenase-2 in heme degradation in *Hmox1*^{-/-} mice. This is because the decrease in circulating bilirubin exceeded that of hematocrit and whole blood hemoglobin in *Hmox1*^{-/-} mice by ~3-fold, compared with *Hmox1*^{+/+} mice (Table 1). Thus, the precise site and mechanism of heme degradation in *Hmox1*^{-/-} mice remain to be established.

The current study raises the intriguing question of how near normal erythropoiesis is maintained in Hmox1 defi-

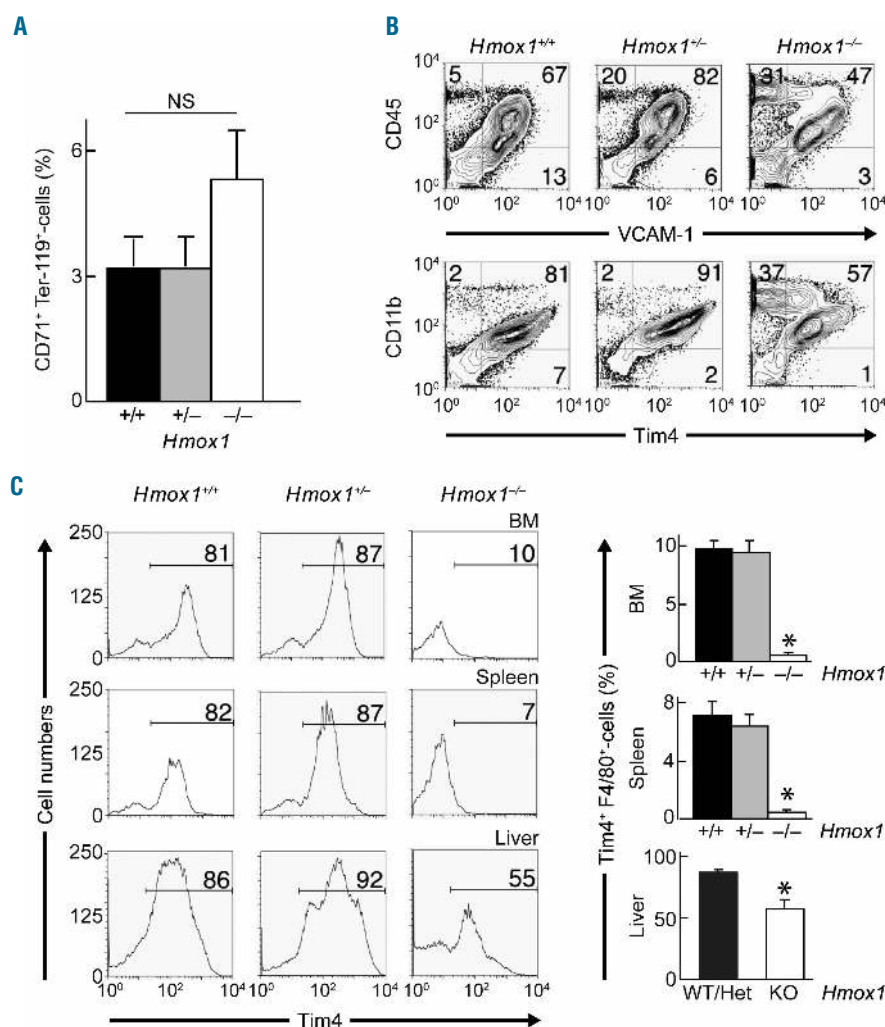


Figure 6. Hepatic macrophages are less severely affected by Hmox1 deficiency than bone marrow and splenic macrophages. (A) Erythropoiesis in the liver of *Hmox1*^{-/-} (n = 3) and *Hmox1*^{+/-} mice (n = 3), as assessed by the frequency of CD71⁺Ter-119⁺ erythroblasts. (B) VCAM-1 and Tim4 expression on hepatic macrophages in *Hmox1*^{+/+}, *Hmox1*^{+/-} and *Hmox1*^{-/-} mice. Representative cytometric data are shown from three separate experiments. (C) Graphic representation of flow cytometric data of BM, spleen and liver cells of *Hmox1*^{+/+} (n = 10), *Hmox1*^{+/-} (n = 5) and *Hmox1*^{-/-} mice (n = 8) gated for F4/80⁺ and examined for Tim4 expression. *P < 0.05 relative to *Hmox1*^{+/+} (BM and spleen) or compared with *Hmox1*^{+/-} plus *Hmox1*^{-/-} (liver).

ciency despite the almost complete absence of BM EBI. Interestingly, efficient enucleation has been reported to take place in the absence of macrophages during the *in vitro* differentiation of hematopoietic stem and progenitor cells.⁵⁰ Macrophages may not, therefore, be absolutely essential for *in vivo* erythropoiesis, although small numbers of VCAM-1⁺ and Forssman ligand⁺ macrophages remain present in the BM of *Hmox1*^{-/-} mice. Some erythropoiesis takes place in the spleen, as demonstrated by the presence of erythroblasts in *Hmox1*^{+/-} and *Hmox1*^{-/-} mice. Indeed, spleen size and proportion of splenic erythroid cells are increased in *Hmox1*^{-/-} compared with control mice, possibly compensating for the defect in BM erythropoiesis. However, in the *Hmox1*^{-/-} spleen erythroblasts are decreased profoundly relative to other erythroid cells and the majority of erythroid cells do not express $\alpha 4\beta 1$ integrin, suggesting that stress erythropoiesis is not the major form of compensatory RBC production in these animals. Moreover, splenic erythropoiesis deteriorates as *Hmox1*^{-/-} mice age and their spleens become increasingly fibrotic. Clearly, additional investigations aimed at answering some of the questions our study raises are warranted, as they will provide novel insights into basic mechanisms governing the formation and removal of

RBC. As well as advancing our knowledge, such studies may provide novel treatment options for the rare cases of HMOX1 deficiency and the anemia of diseases such as chronic inflammatory diseases characterized by abnormal heme turnover and iron recycling.

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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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