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

Stuart T. Fraser,*† Robyn G. Midwinter,† Lucy A. Coupland,‡ Stephanie Kong,‡ Birgit S. Berger,§ Jia Hao Yeo,¶ Oswaldo Cooley Andrade,‖ Deborah Cromer,‖ Cacang Suarna,‡§ Magda Lam,‖° Ghassan J. Maghzal,‖§ Beng H. Chong,‡ Christopher R. Parish,‡ and Roland Stocker†,‡,§

1Laboratory for Blood Cell Development, School of Medical Sciences (Physiology, Anatomy & Histology), Sydney Medical School, The University of Sydney; 2Centre for Vascular Research, School of Medical Sciences (Pathology) and Bosch Institute, The University of Sydney; 3The John Curtin School of Medical Research, The Australian National University, Canberra; 4Vascular Biology Division, Victor Chang Cardiac Research Institute, Darlinghurst; 5Complex Systems in Biology Group, Centre for Vascular Research, University of New South Wales, Kensington; 6School of Medical Sciences, Faculty of Medicine, University of New South Wales, Kensington; and 7Department of Medicine, St George Clinical School, University of New South Wales, Kogarah, Australia

*STF and RGM contributed equally to this work.

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 erythroblast 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 phosphahtidylserine receptor Tim4 in bone marrow, spleen and liver. Heme oxygenase-1 deficiency increases spleen weight and Ter119-erythroid cells in the spleen, although c481-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 erythroblast 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.13,14 Moreover, in Hmox1–/– mice, initial splenic enlargement progresses to red pulp fibrosis, atrophy, and functional hyposplenism in animals older than 9 months,15 reminiscent of the asplenia in the extremely rare HMOX1-null patients who also present with anemia, microcytosis and abnormal iron metabolism.16,17,18 Furthermore, polymorphisms in the HMOX1 gene promoter which can affect the extent of gene transcription are associated with a
range of clinical pathologies, including idiopathic recurrent miscarriage, fetal hemoglobin expression in Brazilian patients with sickle cell anemia, and pre-eclampsia. Splenic macrophages are central to whole body iron recycling and return the iron from cleared RBC to the BM for use in erythropoiesis. 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. Hmox1 is expressed in splenic macrophages and is up-regulated in other cell types in response to heme and oxidative stress. Splenic macrophages are significantly decreased in mice lacking Hmox1, resulting in iron redistribution from the spleen and hepatic Kupffer cells to hepatocytes and proximal tubular cells of the kidney. 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. 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. Moreover, mice with a hematopoietic system reconstituted from Hmox1−/− donor animals demonstrate decreased stress erythropoiesis in response to anemia.

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−/− BM to form EBI. Hmox1−/− 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. 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 erythoblastic islands

EBI were obtained from BM as described elsewhere, 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, 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. Heparinized blood was centrifuged and cells washed and incubated for 10 min at 37°C with hydrogen peroxide (0–150 µM). 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, 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 chimera 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. 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 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. Hematologic parameters of Hmox1−/− animals have not been reported previously even though a decrease in Hmox1 is associated with vascular and hematopoietic
Increased and bilirubin decreased respectively, as such information is currently lacking. We determined Hmox1 and end-product of heme catabolism, respectively, as such information is currently lacking. We also determined important in regulating hemoglobin clearance, although this was not investigated further. We observed that plasma hemoglobin and heme were increased and bilirubin decreased in Hmox1 mice compared with Hmox1 mice (Figure 1B). We therefore asked if Hmox1 deficiency causes a decrease in whole blood hemoglobin. During erythroid maturation, erythroid progenitors interact with the central macrophages of EBI. 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+ and Hmox1+ macrophages and their companions 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

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–/– mice 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–/– mice generally appeared more pigmented than their counterparts from Hmox1+ mice (Figure S1). We next subjected Ter-119+ 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. Hmox1 deficiency caused a blockade of erythropoiesis in the R1 stage, leading to a significant decrease in R3 (Figure 1B). Similar results were observed when the different erythroid developmental stages were segregated using expression of Ter-119, the surface molecule CD44 and cell size

Table 1. Characteristics of mice with Hmox1 deficiency.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Hmox1+/+ (n=11)</th>
<th>Hmox1+–/– (n=8)</th>
<th>Hmox1–/– (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen weight (mg)</td>
<td>112 ± 4</td>
<td>113 ± 7</td>
<td>479 ± 27*</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>47.0 ± 0.7</td>
<td>47.0 ± 1.7</td>
<td>39.3 ± 1.6*</td>
</tr>
<tr>
<td>Whole blood hemoglobin (g/dL)</td>
<td>16.1 ± 0.3</td>
<td>16.4 ± 0.3</td>
<td>12.9 ± 0.2*</td>
</tr>
<tr>
<td>RBC numbers (x 10^12/L)</td>
<td>114 ± 0.3</td>
<td>119 ± 1.0</td>
<td>13.5 ± 1.5*</td>
</tr>
<tr>
<td>Red blood cell diameter (µm)</td>
<td>6.3 ± 0.1</td>
<td>6.3 ± 0.1</td>
<td>5.6 ± 0.1*</td>
</tr>
<tr>
<td>Mean corpuscular volume, (µL)</td>
<td>39 ± 1</td>
<td>41.9 ± 1.5</td>
<td>32.4 ± 1.7*</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin, (pg)</td>
<td>16.2 ± 1</td>
<td>13.6 ± 1</td>
<td>11.2 ± 0.9*</td>
</tr>
<tr>
<td>Plasma erythropoietin (pg/mL)</td>
<td>104 ± 8</td>
<td>100 ± 7</td>
<td>148 ± 14*</td>
</tr>
<tr>
<td>Plasma hemoglobin (µg/mL)</td>
<td>78 ± 3</td>
<td>227 ± 8*</td>
<td>579 ± 238*</td>
</tr>
<tr>
<td>Plasma bilirubin (µmol/L)</td>
<td>0.5 ± 0.04</td>
<td>0.9 ± 0.3</td>
<td>2.2 ± 0.5*</td>
</tr>
<tr>
<td>Plasma heme (µmol/L)</td>
<td>1.9 ± 0.6</td>
<td>2.2 ± 0.3</td>
<td>0.7 ± 0.1*</td>
</tr>
</tbody>
</table>

Individual parameters were 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.
examined the expression of α4β1-integrin on BM Ter-119+ erythroid cells, as erythroid cells express Hmox1, α4-integrin regulates terminal erythroid maturation, and α4β1-integrin interaction with macrophage vascular cell adhesion molecule 1 (VCAM-1) is a key determinant of EBI formation. Ter-119+ cells of Hmox1–/– mice expressed higher levels of α4β1-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. BM from Hmox1–/– mice had significantly decreased numbers of EBI macrophages expressing the surface antigens F4/80, VCAM 1, CD11b, Ly6C, CD119 and ER-HR3 (Figure 3B). Consistent with this observation, the expression of the glycosphingolipid Foresman antigen, a specific marker for EBI-macrophages, was decreased profoundly in BM macrophages of Hmox1–/– compared with Hmox1+/+ and Hmox1+/– mice (Figure 3C). These results indicate that BM of Hmox1–/– mice has 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 α4-integrin Ter-119+ cells (Figure 4B). The latter include both erythroblasts and reticulocytes. Compared with Hmox1+/+, circulating Ter-119CD71+ 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...
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 α4-integrin as well as α5-integrin. The latter regulates the interaction of stress erythroblasts with the splenic microenvironment. The expression of both α4- (Figure 4F) and α5-integrin (data not shown) in splenic erythroblast 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. Oxidized Prx2 is a thiol-dependent peroxidase essential for maintaining the lifespan of RBC in mice, and the third most abundant RBC protein. 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. An increase in the ratio of Prx2 dimers to monomers reflects increased oxidative stress.

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 study 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 oxidized Prx2 as dimers compared with cells from Hmox1+/- mice (Figure 5B), consistent with a previous study 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 oxidized 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. 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
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, 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. Free heme is a strong oxidant and cytotoxic, and the inability of Hmox1-deficient splenic macrophages to toler-
Heme oxygenase-1 deficiency and erythropoiesis

Figure 4. Absence of Hmox1 alters circulating and splenic erythroid population frequency. Hmox1 deficiency increases circulating erythroid cells that stain with (A) DRAQ5, (B) the erythroblast marker α4β1 integrin, and (C) CD71 (n = 5, 3 and 6 for Hmox1+/+, Hmox1+/− and Hmox1−/− mice, respectively). (D) Increased frequency in Ter-119+ erythroid cells in the spleen of Hmox1+/+ (n = 5) compared with Hmox1+/− (n = 6) and Hmox1−/− (n = 3) mice. (E) Splenic erythroid maturation in Hmox1+/+ (black, n = 6), Hmox1−/−/− (gray, n = 3) and Hmox1−/− mice (white, n = 5) as assessed by expression of Ter-119+ and CD71. (F) Decreased frequency of Ter-119+ erythroblast cells expressing α4β1 integrin (left panel), α4β1high (black) or α4β1low integrin (white) (right panel). Data show representative flow cytometric profiles and their quantification (n = 3–10 for different groups). *P<0.05 compared with Hmox1+/+ and Hmox1−/− mice.

ate heme leads to their death and disruption of the iron recycling process. Here, we show that Hmox1 also plays significant roles in regulating erythroid cell-macrophage interactions in the BM.

BM from mice lacking Hmox1 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 α4β1 integrin on neighboring erythroblasts. During erythroid cell maturation, the nucleus becomes condensed and expelled, surrounded by a thin layer of hemoglobin-containing cytoplasm and encapsulated in an erythroid cell membrane replete with α4β1 integrin. We propose that these hemoglobin-containing, expelled nuclei are engulfed preferentially by VCAM-1+ macrophages, leading to Hmox1 induction seen in wild-type EBI macrophages (Figure 2A). We further propose that an inability to catabolize the heme present in ‘pyrenocytes’ leads to the selective death of VCAM-1+ macrophages in Hmox1 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 Hmox1 deficiency increases and Hmox1 induction decreases VCAM-1 expression. 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 Hmox1−/− mice, we observed highly abnormal EBI macrophages with domed morphology rather than flattened, spread-out morphology. These EBI also lack clear interactions with erythroblasts. Thus, normal EBI formation no longer appears possible in Hmox1 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. 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 Hmox1−/− animals (increased plasma hemoglobin for example) are involved in the pathologies observed in patients with HMOX1 polymorphisms.

Our studies provide direct evidence that Hmox1 deficiency increases RBC lifespan, likely by attenuating macrophage-mediated RBC removal in the spleen, liver and BM by decreasing both macrophage numbers and
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 Prx2 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 elevation plasma concentrations of the pro-oxidant heme.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,12,30 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-
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. 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–/+ 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 α4β1 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.

Acknowledgments
This work was supported by a program grant (BHC, CRP, RS) and Senior Principal Research Fellowship (RS) from the Australian National Health and Medical Research Council, the Office of Health and Medical Research, NSW State Government, and the Bosch Institute Translational Grant-in-Aid and Sydney Medical School New Staff grant (both STF). JHY was supported by a NWG Macintosh Scholarship. We thank Dr Louise Cole for her assistance and help with confocal imaging, and acknowledge support from the Bosch Institute Live Cell Analysis, Advanced Microscopy and Mass Spectrometry Core Facilities and the Australian Center for Microscopy & Microanalysis at the University of Sydney.

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.
References


S.T. Fraser et al.