Minihepcidins improve ineffective erythropoiesis and splenomegaly in a new mouse model of adult β-thalassemia major

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Supplemental Methods

Drug preparation

MH was prepared fresh for each administration.(1) The drug was formulated in ethanol 100%, SL-220 (SUNBRIGHT) and water at desired concentration. The ethanol concentration was 10% of the final volume injected. All animals were treated with either Vehicle control or MH (5.25 mg/kg or 2.625 mg/kg) by subcutaneous injections (SC) every other day for six weeks and data were collected 1.5 days after the last injection.

ROS analysis

To study the ROS production in the erythroid compartment of bone marrow and spleen, 1 x 10^6 cells per reaction were first incubated with a PE/Cy7 anti-mouse/human CD44 (Biolegend #103030) and an APC conjugated anti-mouse Ter119 antibodies (BD-Biosciences-Pharmingen #561033) for 15 min on ice and in the dark. Cells were then washed with PBS and stained for ROS detection using the indicator CM-H2DCFDA (Invitrogen #C6827) for 15 min at 37^C, 5% CO_2 in the dark. For all the analyses, cells were sorted using a FACS Calibur instrument (BD) and the results were analyzed with FlowJo software (Tree Star Inc.).

Measurement of tissue iron content and serum iron parameters

Serum parameters [iron and transferrin saturation (TSAT)] were measured using the Iron/TIBC Reagent Set (BioPacific Diagnostic). Serum erythropoietin (EPO) and hepcidin (HAMP) were analyzed by ELISA according to the manufacturer's instructions [Mouse Erythropoietin Quantikine kit (R&D Systems) and Hepcidin-Murine-Compete kit (Intrinsic LifeSciences), respectively)]. We excluded that MH cross-reacts with the serum HAMP elisa assay (used to quantify the endogenous mouse hepcidin) by injecting MH into *Hamp*-KO mouse and analyzing the serum HAMP levels. No differences were observed in HAMP levels comparing the serum of a mouse treated with MH and a control *Hamp*-KO animal after 1, 3, 6 and 20 hour following MH administration. The non-heme iron concentration of the wet liver, spleen, and heart was determined using a colorimetric assay.

Pathology and Peripheral Blood smear

Perls' Prussian blue staining of mouse liver, spleen and kidney sections and Giemsa staining of peripheral blood smear were performed using the CHOP Pathology Core Facility.(1) Images were captured using a Leica DM4000B upright microscope paired with a Spot RT/SE Slider camera (103/numerical aperture 0.20 objective) using objectives 20x/N.A. 0.70, 40x/N.A. 0.85 and 63x/N.A. 0.90. Images were then acquired using the Spot 5.1 software.

Hemichrome analysis

To visualize membrane-bound globins (hemichrome) we utilized urea gel electrophoresis (TAU gel) as previously described.(1, 2)
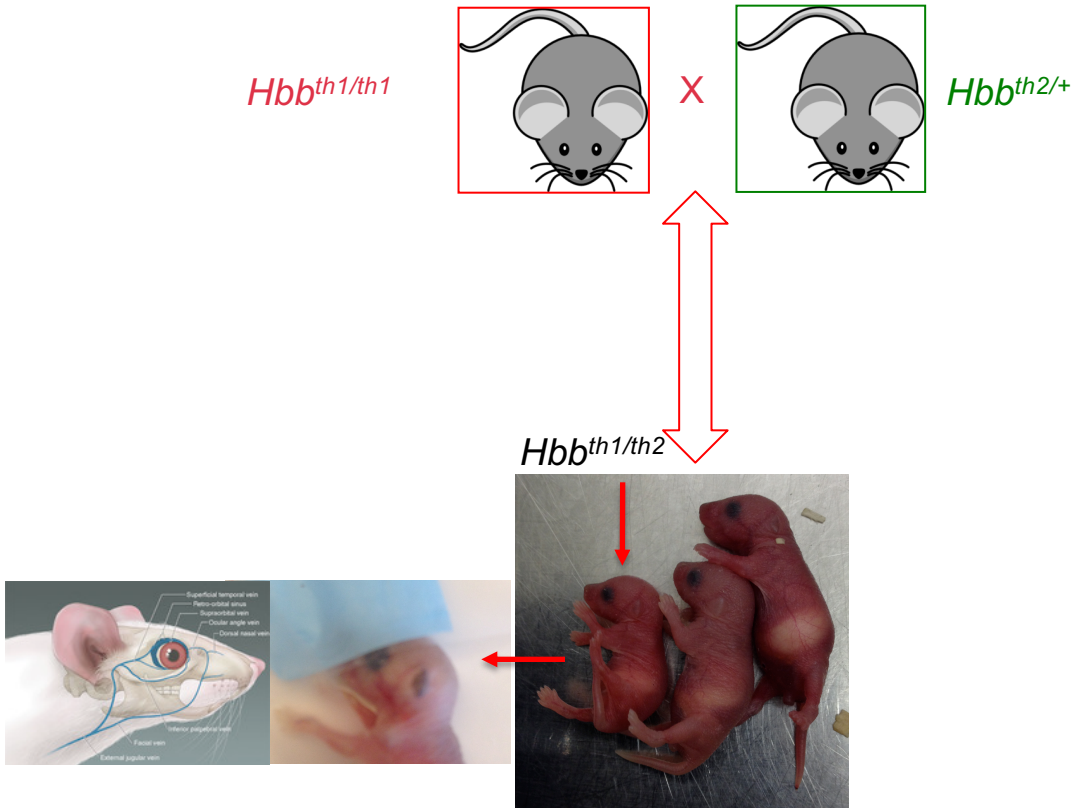
Fluorescence-activated cell sorter analysis

Analysis of erythropoiesis in the bone marrow (BM) and spleen was performed as previous described.(2) Single cell suspensions were sorted using a FACSCalibur (BD-Biosciences) and the results analyzed with FlowJo software (Tree Star).
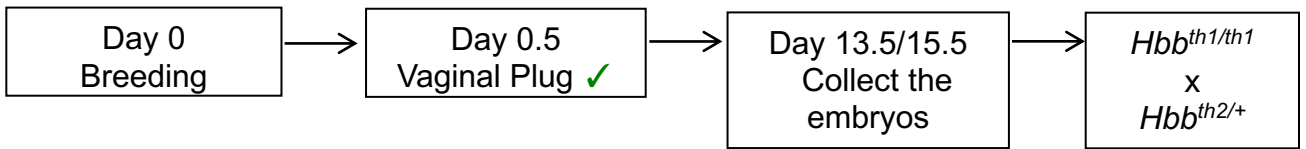
Supplemental References

1.   Casu C, Oikonomidou PR, Chen H, Nandi V, Ginzburg Y, Prasad P, et al. Minihepcidin peptides as disease modifiers in mice affected by beta-thalassemia and polycythemia vera. Blood. 2016 Jul 14;128(2):265-76.

2.   Guo S, Casu C, Gardenghi S, Booten S, Aghajan M, Peralta R, et al. Reducing TMPRSS6 ameliorates hemochromatosis and beta-thalassemia in mice. J Clin Invest. 2013 Apr;123(4):1531-41.

A

B

Day 0 Breeding	→	Day 0.5 Vaginal Plug ✓	→	Day 13.5/15.5 Collect the embryos	→	$Hbb^{th1/th1}$ x $Hbb^{th2/+}$

Supplemental Fig.1 Strategies to generate ß-thalassemia major model. (A) We intercrossed two mouse model of ß-thalassemia intermedia, $Hbb^{th1/th1}$ and $Hbb^{th2/+}$. Pups with the experimental genotype were alive after birth (marked with the red arrow) but did not to survive more than few hours. Pups did not survive even after facial vein blood transfusion or IP blood transfusion. (B) Strategy to obtain the desired model via engraftment of fetal liver cells.
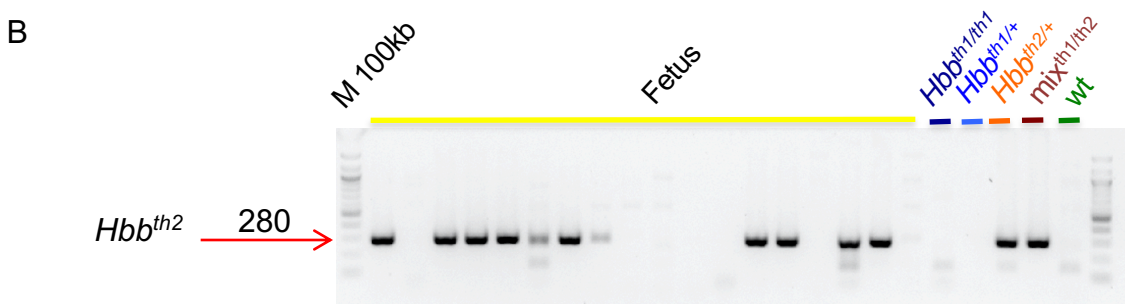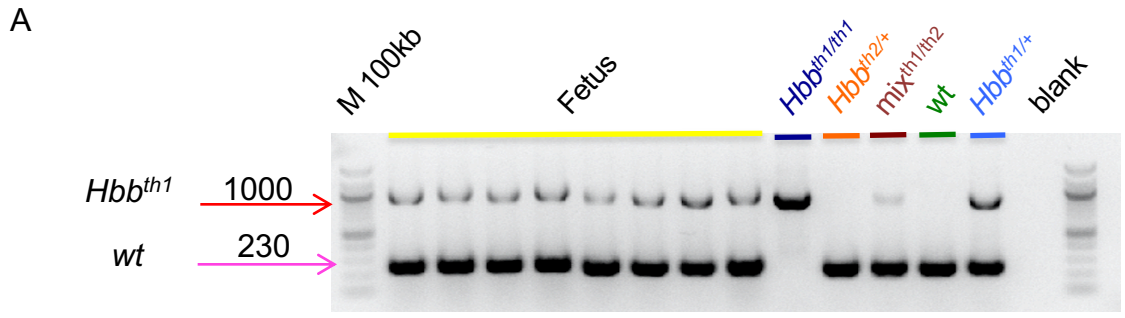
Supplemental Fig.2 Selection of the positive fetuses. Positive fetuses were identified by PCR. For the screening of the mice we used specific primers that showed different bands for each genotype. (A) $Hbb^{th1/th1}$ specific primers amplified a band of 1000bp, while the WT specific primers amplified a band of 230bp. (B) $Hbb^{th2/+}$ primers amplified a band of 280bp.

| Genotype		Primers Sequences
Hbb^{th1/th1}	3823 3824 3821 3822	5'- TGG CAA GTG TCC CTA AGA CC-3' -MUT 5'- GTG TAA AGG TCC CAG GCA AA-3' -MUT 5'- CGA AGC CTG ATT CCG TAG AG-3' -WT 5'- TCA TCG AGG TTC ACC TTT CC-3' -WT
Hbb^{th2/+}	6916 6917 8744 8745	5'- CTT GGG TGG AGA GGC TAT TC-3' -MUT 5'- AGG TGA GAT GAC AGG AGA TC-3' -MUT 5'- CAA ATG TTG CTT GTC TGG TG-3' -Int-Contrl 5'- GTC AGT CGA GTG CAC AGT TT-3' -Int-Contrl

Supplemental table 1. Primers sequences.

	PCR-Protocols
Hbb^{th1/th1}	*Hbb*^{th2/+}
step 1: 94^{0}C 2' step 2: 94^{0}C 20'' step 3: 65^{0}C 15'' (-0.5 each cycle) step 4: 68^{0}C 10'' steps 2 to 4 repeated 10 times step 5: 94 ^{0}C 15'' step 6: 60^{0}C 15'' step 7: 72^{0}C 10'' step 8 72^{0}C 2' step 9: 4^{0}C ∞	step 1: 95^{0}C 3' step 2: 95^{0}C 15'' step 3: 58^{0}C 15'' step 4: 72^{0}C 20'' steps 2 to 4 repeated 40 times step 5: 72^{0}C 5' step 6: 4^{0}C ∞

**Supplemental table 2. Genotyping protocols for the screening of *Hbb*^{th1/th2} embryos.**

A

B
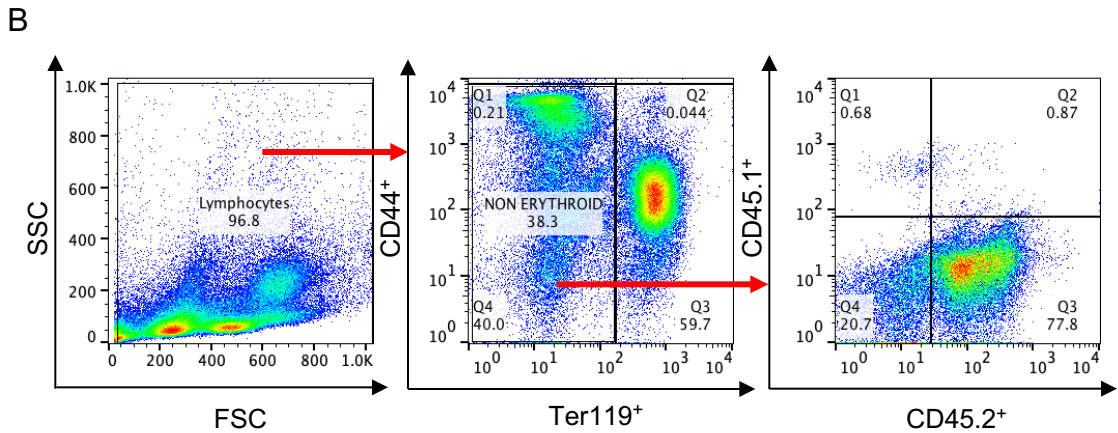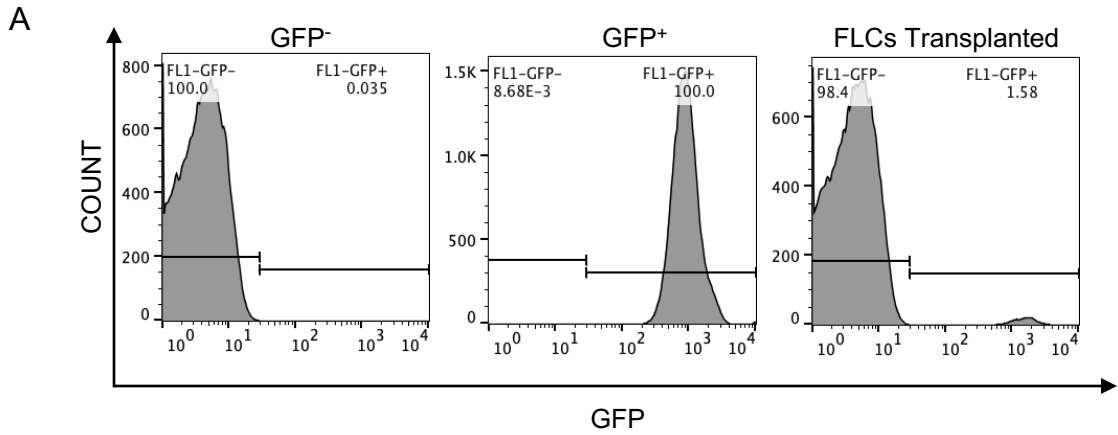
**Supplemental Fig.3 Assesment of chimerism levels by flow cytometry analysis.**
(A) Peripheral blood GFP expression in FLCs (Fetal Liver Cells) transplanted GFP$^+$ recipient mice.
(B) Bone marrow CD45.1 versus CD45.2 expression in FLCs-transplanted CD45.1 recipient mice.
Animals showing chimerism above 3% were not included in the analysis.
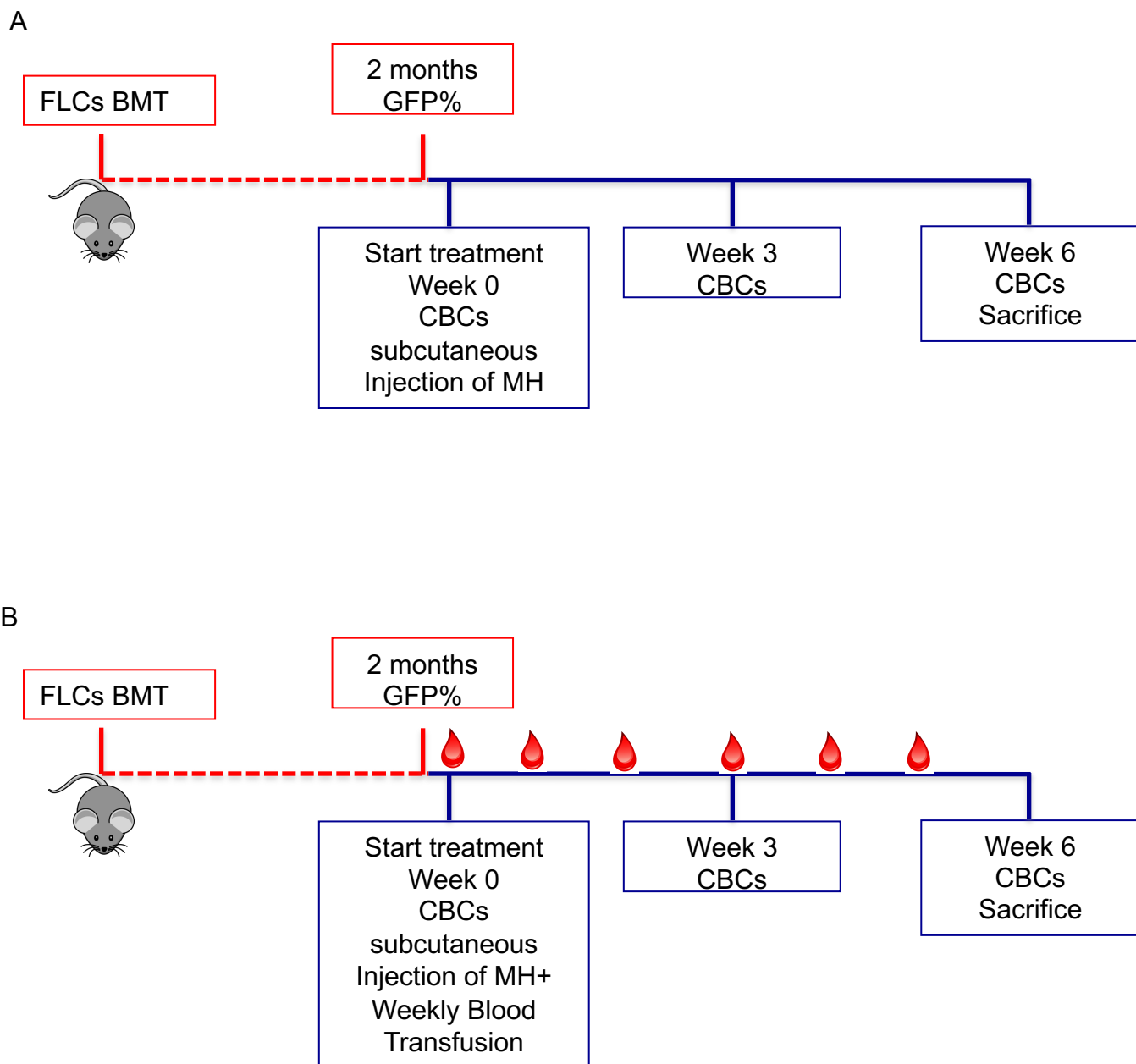
Supplemental Fig.4 Experimental design. Administration of MH in absence (A) or presence (B) of blood transfusion in FLCs BMT mice. Complete blood count (CBC) was performed at time point 0, week 3 and at the end point.
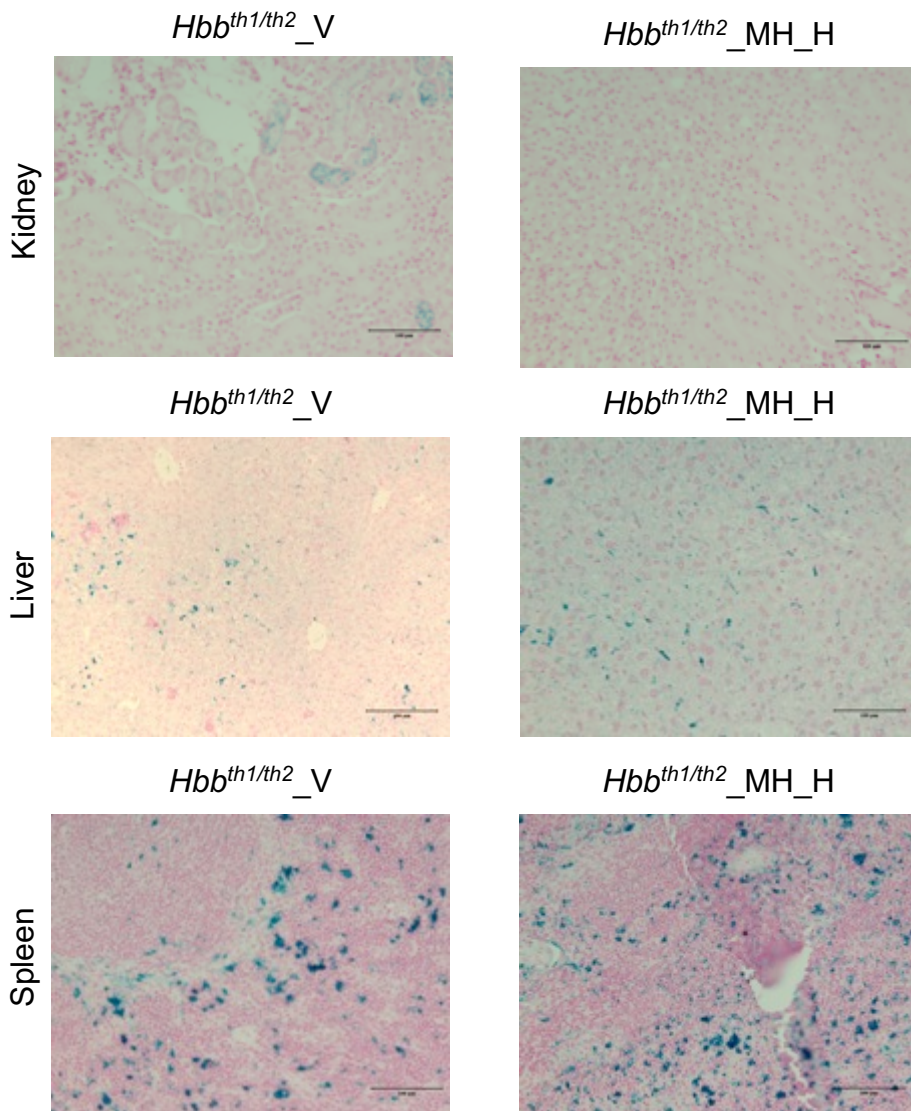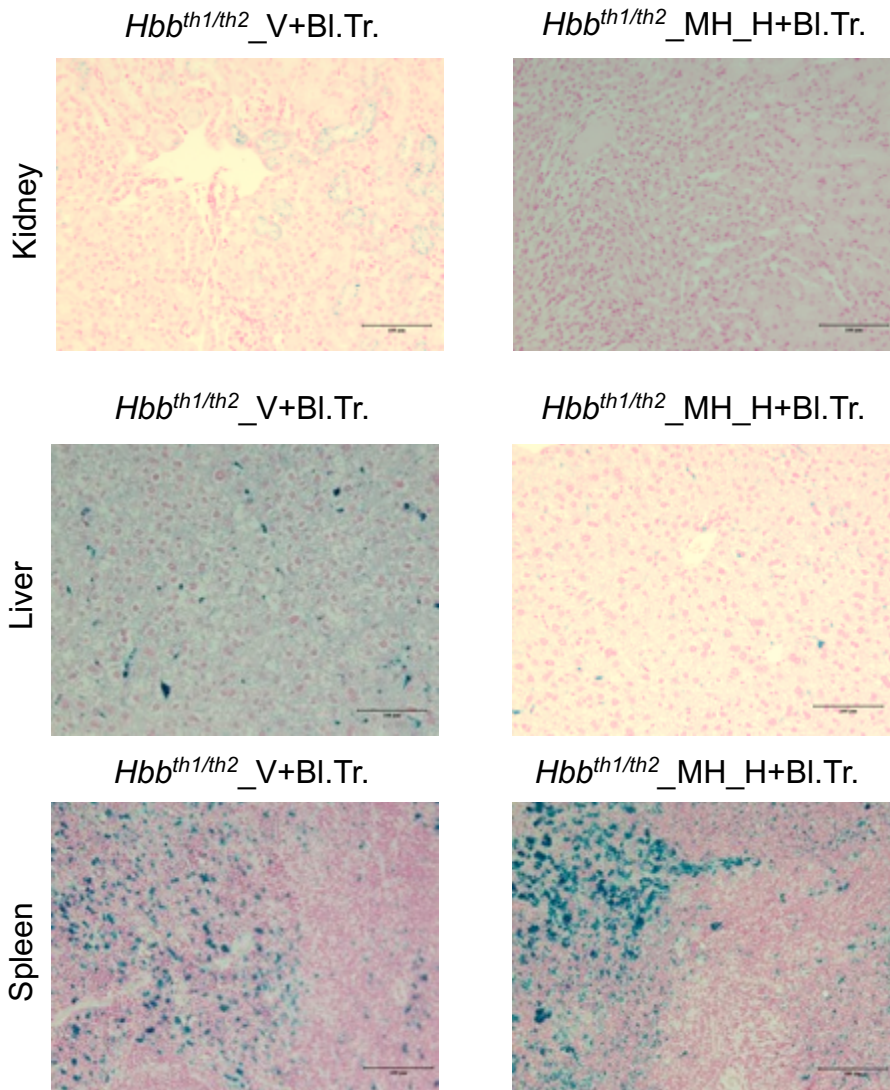
Supplemental Fig.5 Iron content in liver, kidney and spleen of *Hbb^{th1/th2}-BMC* mice treated with MH. Perl's Prussian blue staining on kidney, liver and spleen sections of *Hbb^{th1/th2}-BMC* (Bone Marrow Chimeras) mice.
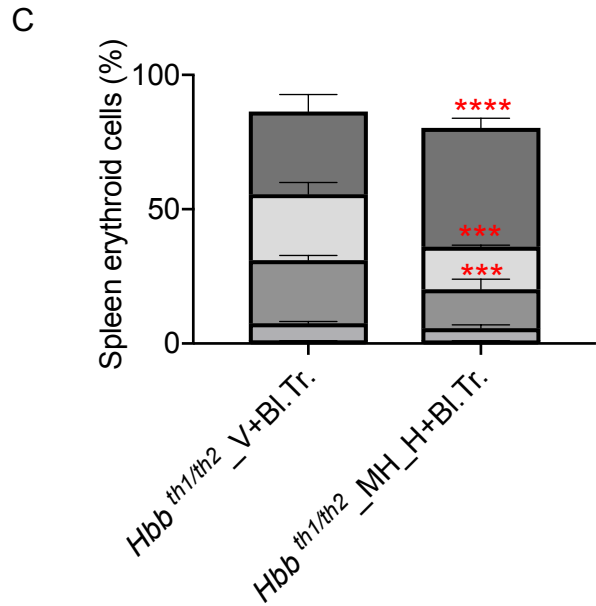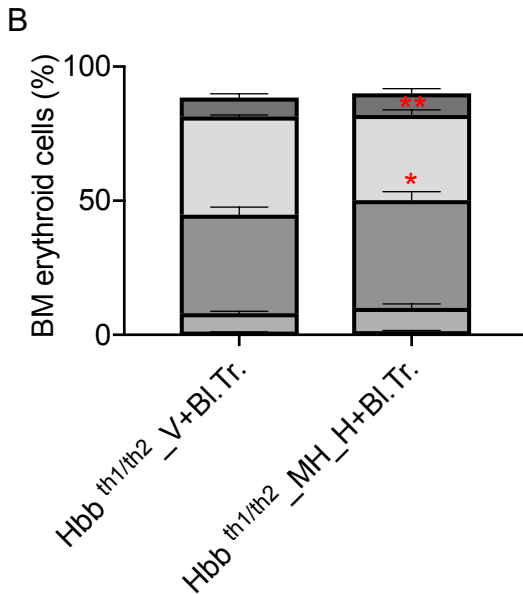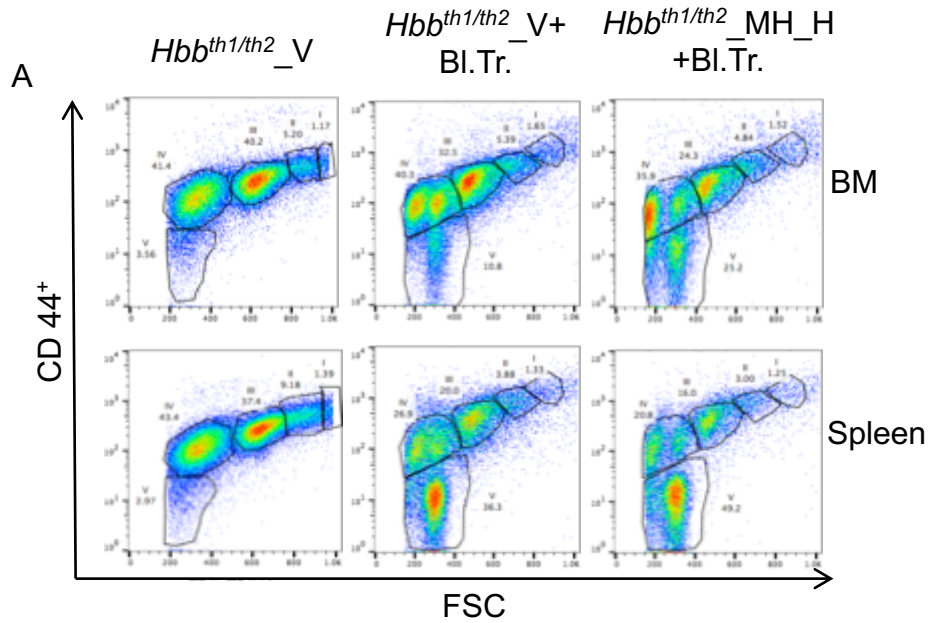
ProErytroblast   *Basophilic E.*   *Polycromatic E.*   *Ortho/Reticulocytes*   *Erythrocytes*

Supplemental Fig.6 Effect of treatment with MH co-operated with blood transfusion in *Hbb^th1/th2^-BMC* mice on the percentage of mature and immature erythroid cells. Based on the percentage of mature and immature erythroid cell populations, the effect of treatment with MH is complementary to that of blood transfusion (Bl.Tr) in *Hbb^th1/th2^-BMC* mice. (A)FACS analyses using CD44$^+$ (or Ter119$^+$-CD71$^+$) cells demonstrate that MH improves erythropoiesis in the BM and spleen of *Hbb^th1/th2^-BMC* mice receiving blood transfusion. (B) Percentage of BM and (C) spleen erythroid cells (n=4-5 animals per group). Results are presented as means ± SD: ****P≤.001, ***P≤.005, **P≤ .01; *P≤ .05.

Supplemental Fig.7 Iron analysis in *Hbb^{th1/th2}-BMC mice* treated with MH and blood transfusion. Perl's Prussian blue staining on kidney, liver and spleen sections of *Hbb^{th1/th2}-BMC* mice blood transfused.
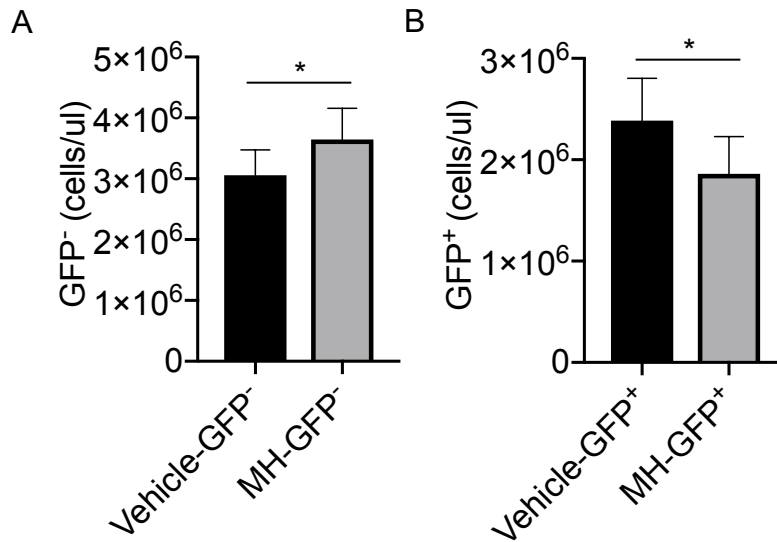
Supplemental Fig.8 Effect on RBCs of reduced transfusion regiment in combination with MH.
Hbb^{th1/th2}-BMC mice received MH_H treatment for six weeks and blood transfusion for the first 3 weeks of treatment (300ul of GFP⁺ cells once at week). At the end of the treatment, in the animals treated with transfusion and MH, the concentration of endogenous RBCs (GFP⁻) increased, similarly to that found in non-transfused animals treated with MH (A). However, in the animals treated with transfusion and MH, we observed a decrease in the absolute number of the transfused RBC (GFP⁺) compared to those treated with transfusion alone (B). Results are presented as means ± SD: *P≤ .05.