Cell Biology

High-altitude hypoxia exposure inhibits erythrophagocytosis by inducing macrophage ferroptosis in the spleen

Reviewed Preprint

Published from the original preprint after peer review and assessment by eLife.

About eLife's process

Reviewed preprint version 2
September 1, 2023

Reviewed preprint version 1
May 23, 2023 (this version)

Posted to preprint server
March 25, 2023

Sent for peer review
March 23, 2023

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Abstract

High-altitude polycythemia (HAPC) occurs in high-altitude (HA) environments and involves an imbalance between erythropoiesis and eryptosis. Spleen/splenic macrophages are an important primary tissue/cell of eryptosis and iron recycling. However, the role of the spleen in the pathogenesis of HAPC and the effect of hypobaric hypoxia (HH) on the biology of the spleen and splenic macrophages are still unclear. We used a mouse hypobaric hypoxia (HH) exposure model to simulate an *in vivo* study of 6000 m HA exposure. For *in vitro* studies, we used a primary splenic macrophage model treated with 1% hypoxia. We found that the HH-treated mouse model promoted erythropoiesis and led to erythrocytosis. In addition, HH exposure resulted in marked splenic contraction followed by splenomegaly for up to 14 days. HH exposure impaired the red blood cell (RBC) handling capacity of the spleen, which was caused by a decrease in splenic macrophages in the red pulp. Moreover, HH treatment for 7 and 14 days promoted iron mobilization and ferroptosis in the spleen, as reflected by the expression of metabolism-related proteins and ferroptosis-related proteins. All of the protein expression levels were similar to the gene expression levels in human peripheral blood mononuclear cells. Single-cell sequencing of the spleen further demonstrated a significant decrease in macrophages in the spleen 7 days after HH exposure. In *in vitro* studies, we confirmed that primary splenic macrophages decreased and induced ferroptosis following hypoxic treatment, which was reversed by pre-treatment with the ferroptosis inhibitor ferrostatin-1. Taken together, HH exposure induces splenic ferroptosis, especially in red pulp macrophages, which further inhibits the clearance of RBCs from the spleen. As such, it promotes the retention of RBCs in the spleen and causes splenomegaly, which may further lead to the persistent production of RBCs and ultimately to the development of HAPC.

eLife assessment

The authors set out to study the development of high altitude polycythemia, which affects mice in a hypoxia chamber and humans staying at a hypoxic atmosphere at high altitude. The findings are **useful** both for initiating the discussion of the hypothesis that splenic red pulp macrophages, central to red cell survival, are impacted by hypoxia, and for providing some data that partially supports this hypothesis. However, the current data are **inadequate** to fully support the authors' conclusions.

Background

A plateau is a special environment characterized by low atmospheric pressure and low partial oxygen pressure. Long-term exposure to plateau environments may lead to chronic mountain disease [1]. High-altitude polycythemia (HAPC) is a common and widespread chronic mountain sickness characterized by excessive erythrocytosis [2]. Hypobaric hypoxia (HH) is the main cause of erythrocytosis to alleviate hypoxic conditions in tissues under high-altitude (HA) exposure [3]. In a healthy organism, erythropoiesis in bone marrow is equivalent to eryptosis in the spleen [4]. Although HA/HH exposure can affect the balance between the formation and clearance of RBCs, a new physiological RBC steady state can be rapidly formed in normal individuals [5]. Nevertheless, RBC homeostasis is disturbed and never formed into a homeostasis status in HAPC patients, which finally results in a continuous increase in RBCs [4]. It is well known that HA/HH exposure may lead to erythropoiesis; however, the pathogenesis of eryptosis under HA/HH conditions remains unclear.

The spleen clears the altered RBCs in the circulation system, which helps to balance erythropoiesis and RBC clearance [6]. Moreover, red pulp macrophages (RPMs) of the spleen are the major scavengers that remove unhealthy, old, and malformed RBCs from the circulation to recover iron [7]. In brief, RPMs recognize the RBCs to be removed through the signal on their cell surface and trigger RBC endocytosis and digestion into heme in lysosomes [8]. Subsequently, the heme is decomposed by heme oxygenase-1 (HO-1) into biliverdin, carbon monoxide and iron [9]. Most of the iron recycling from heme is transported out of the cell through ferroportin (Fpn) and then participates in the formation of RBCs through the combination of transferrin (Tf) and transferrin receptor (TfR) on the cell membrane [10]. A portion of the released iron is loaded into cellular ferritin (Ft; including Ft-H and Ft-L). Ft-H oxidizes Fe^{2+} (ferrous ion) to Fe^{3+} (ferric ion) in the presence of O_2, and then Fe^{3+} can be stored on Ft-L [11]. When the body's iron metabolism is vigorous, nuclear receptor coactivator 4 (NCOA4) can recognize Ft-H, bring Ft into the lysosomal pathway for degradation, and release iron in Ft for iron recycling in the body [12]. However, some iron is free in cells in the form of ferrous iron (Fe^{2+}), which is well known to be an important initiator of free radical oxidation. Moreover, the accumulation of large amounts of Fe^{2+} ions in cells may cause ferroptosis [13].

Although erythropoiesis under HA/HH is well studied thus far, the precise effects of HA/HH on erythrophagocytosis in the spleen remain largely unexplored. Based on the fact that the spleen is the major organ for RBC processing and that RPMs play an important role in recycling iron from RBC clearance, we investigated the effects of HA exposure on spleen/splenic macrophages in an HH-exposed mouse model (6000 m exposure conditions). In the current study, we further explored whether HA/HH could regulate the erythrocyte disposal and iron recycling of macrophages, and specifically predict the damage of erythrophagocytosis after HA/HH exposure. We demonstrated that HH exposure induced ferroptosis in the spleen, especially in macrophages, which led to a

decrease in the number of macrophages, followed by eryptosis and iron recycling in the spleen. These findings may be clinically relevant to continuous pathological erythrocytosis and HAPC progression under HA exposure.

Methods

HH mouse exposure model

Male C57BL/6 male mice (at 8 weeks of age) were obtained from the animal experimental centre of Nantong University. Mice were adapted to the facilities for 3 days before experiments. Mouse models of HH exposure were established by placing the mice in the decompression chamber (TOW-INT TECH, ProOx-810, China) for 1 d, 2 d, 3 d, 7 d, and 14 d. The parameters were set as follows: 6000 m above sea level, oxygen partial pressure of 11 kPa, lifting speed of 20 m/s, chamber pressure of 54 kPa, temperature of 25 ℃, humidity of 40%, and 12-h light/dark cycles. Tuftsin (1.5 mg/kg, MCE, HY-P0240, USA) was used to stimulate the phagocytosis of macrophages and was injected at 7 d and 11 d during the 14-d period of mice exposed to HH.

Splenic macrophage culture and treatments

After isoflurane anaesthesia, mice were transcardially perfused with precooled PBS, and then splenic tissue (approximately $1mm^3$ in volume) was collected and separated in cold, phenol red-free Hanks' balanced salt solution (HBSS; Gibco, Grand Island, NY, USA). The cell extracts were centrifuged at 500 rpm for 5 min at 4 ℃. The pellets were then resuspended and incubated with ammonium-chloride-potassium (ACK) lysing buffer (Thermo Fisher Scientific, USA) for 3 min at room temperature. Next, cell extracts were purified in a four-phase Percoll (Sigma−Aldrich, St. Louis, MO, USA) gradient: 0, 30, 40, and 50% in HBSS. After centrifugation at 500 rpm for 20 min at 4°C, the macrophage-enriched fraction was collected at the interface between the 30% and 40% phases. Cells were washed in two rounds with HBSS and then resuspended in AIM V medium (Thermo Fisher Scientific). The cells in this medium were cultured at 37 ℃ and 5% CO_2 for 12 h. After the cells were treated with ferrostatin-1 (Fer-1, 2 μm; Selleck Chemicals, S7243, USA) 1 h in advance, the cells were placed in a hypoxia workstation (Invivo2, Ruskinn, UK) for 24 h.

Blood smear and hematological indices

The collected blood was gently mixed with EDTA (15 g/L) to obtain anticoagulant blood, which was detected by a hematology analyser (XE-5000, Sysmex Corporation, Japan). Blood smear preparation method: a blood sample is dropped onto a slide, and another slide is used to push the blood sample into a uniform thin layer at a constant speed. After the blood samples were dried, Wright's stain solution was added and incubated for 1 min. Then, the slide was rinsed with water. Blood smears were photographed with a DM4000B microscope (Leica, Germany).

Western blot

Tissue and cell samples were homogenized on ice after cell lysates and protease inhibitors were added. Centrifugation was performed at 12,000 rpm for 15 min at 4°C to collect the supernatant. Protein concentration was determined by the BCA detection method. A sample containing 30 μg protein was loaded and run in each well of SDS− PAGE gels. The membranes were incubated with the following antibodies: HO-1 (1:2000, Abcam, ab13243, USA); Ft-L (1:1000, Abcam, ab69090, USA); Ft-H (1:1000, Novus, NBP1-31944, USA); NCOA4 (1:1000, Santa Cruz, sc-373739, USA); TfR (1:1000, Thermo Fisher, 13-6800, USA); Fpn (1:1000, Novus, NBP1-21502, USA); ACSL4 (1:1000, Santa Cruz, sc-271800, USA); xCT (1:1000, Proteintech, 26864-1-AP, USA); Gpx4 (1:1000, Abcam, ab125066, USA); CD206 (1:1000, RD, AF2535, USA); CD16 (1:2000, RD, AF1960, USA); and β-actin (1:1000, Sigma−

Aldrich, A5316, USA). The secondary antibodies were as follows: goat anti-mouse (1:10000, Jackson, USA) and goat anti-rabbit (1:10000, Jackson, USA). The gray value of specific blots was scanned and analysed using ImageJ software (National Institute of Health, USA).

RT−PCR analysis

Total RNA extraction and RT−PCR analysis were performed essentially as described previously [14]. Primer sequences were as follows:

Gene	Primer sequence (5'-3')	Product length (bp)
Csf1	F: GGCTTGGCTTGGGATGATTCT	126
	R: GAGGGTCTGGCAGGTACTC	
Csf2	F: GGCCTTGGAAGCATGTAGAGG	104
	R: GGAGAACTCGTTAGAGACGACTT	
CCL2	F: TTAAAAACCTGGATCGGAACCAA	121
	R: GCATTAGCTTCAGATTTACGGGT	
CCL7	F: GCTGCTTTCAGCATCCAAGTG	135
	R: CCAGGGACACCGACTACTG	

GEO analysis

GSE46480 samples were found in the GEO database of NCBI with the keyword "High Altitude" search. The data set was divided into two groups, one for the plain (Base Camp) and the other for the plateau (Altitude), with a sample size of 98 for each group. Blood samples were collected from the same person at McMurdo Station (48 m) and immediately transferred to Amundsen-Scott South Pole Station (2835 m) on the third day. R language was used for data analysis, and GraphPad Prism was used for statistics. **Malondialdehyde (MDA), Cysteine (Cys) and Glutathione (GSH) content detection** MDA was detected by a Micro Malondialdehyde Assay Kit (Solarbio, BC0025, China). Cys was detected by a Micro Cysteine Assay Kit (Solarbio, BC0185, China). GSH was detected by a Micro Reduced Glutathione Assay Kit (Solarbio, BC1175, China).

Immunofluorescence

Frozen sections of the spleen (9 μm) were stored at −80°C. Sections were placed at room temperature for 30 min before immunofluorescence staining, incubated in 10% BSA-PBS for 10 min, incubated overnight with F4/80-PE (1:200, Biolegend, 123110, USA), washed with 0.05% PBST three times, incubated with DAPI for 10 min, washed with PBS three times, sealed with 50% glycerine-PBS, and photographed with a confocal laser scanning microscope (SP8, Leica Microsystems, Wetzlar, Germany).

Tissue iron staining (DAB-enhanced Perls' staining)

DAB-enhanced Perls' staining for the spleen paraffin sections was performed as described previously [15]. Briefly, the sections were washed with PBS and cultured in freshly prepared Perls' solution (1% potassium ferricyanide in 0.1 M hydrochloric acid buffer). The slides were then immersed and stained with DAB. All slides were counterstained with hematoxylin and visualized under a DM4000B microscope (Leica, Germany). Data were collected from three fields of view per mouse and semiquantitatively analysed with ImageJ software. Quantitative results of iron staining were finally normalized to the NN control group.

Flow cytometry

1) The steps of reticulocyte ratio detection were as follows: The experimental tube and negative control tube were prepared, 125 μL normal saline, 4 μL anticoagulant and 125 μL TO working solution (1 μg/mL, Sigma, 390062, USA) were added to each tube, and 250 μL normal saline and 4 μL anticoagulant were added to each tube of the negative control tube. After incubation at room temperature for 1 h, flow cytometry analysis was carried out by using the FL1 (488 nm/525 nm) channel.

2) The steps for the determination of intracellular divalent iron content and lipid peroxidation level were as follows: The spleen was made into a single cell suspension, and then the RBCs were lysed. This step can be omitted from cell samples. A total of 1×10^6 cells were incubated with 100 μL of BioTracker Far-red Labile Fe^{2+} Dye (1 mM, Sigma, SCT037, USA) for 1 h or C11-Bodipy 581/591 (10 μM, Thermo Fisher, D3861, USA) for 30 min. After the cells were washed with PBS twice, flow cytometry analysis was carried out by using the FL6 (638 nm/660 nm) channel for determination of intracellular divalent iron content or the FL1 (488 nm/525 nm) channel for determination of lipid peroxidation level.

3) The steps for detecting the mortality of spleen cells and peritoneal macrophages were as follows: 1×10^6 cells were incubated at room temperature for 40 min with 2 μM Calcein AM and 8 μM PI. Flow cytometry analysis was carried out by using FL1 (488 nm/525 nm, Calcein AM) and FL3 (488 nm/620 nm, PI) channels.

4) The steps for detecting the number of M1/M2 macrophages in the spleen were as follows: 1×10^6 cells were incubated with 2% mouse serum-PBS for 10 min, incubated with F4/80-PE (1:200), CD86-PE/Cyanine7 (1:20, Biolegend, 105014, USA), and CD206-Alexa 647 (1:80, BD, 565250) for 30 min, and washed with 2% mouse serum-PBS twice. Flow cytometry analysis was carried out by using FL2 (488 nm/575 nm, F4/80-PE), FL5 (488 nm/755 nm, CD86-PE/Cyanine7) and FL6 (638 nm/660 nm, CD206-Alexa 647) channels.

5) The steps for detecting the number of monocytes in blood, spleen and bone marrow were as follows: 1×10^6 cells were incubated with 2% mouse serum-PBS for 10 min, incubated with F4/80-PE, CD11b-PE/CY7 (1:2000, BD, 552850, USA), and Ly6C-APC (1:2000, Thermo Fisher, 17-5932-82) for 30 min, and washed with 2% mouse serum-PBS twice. Flow cytometry analysis was carried out by using FL2 (488 nm/575 nm, F4/80-PE), FL5 (488 nm/755 nm, CD11b-PE/CY7) and FL6 (638 nm/660 nm, Ly6C-APC) channels.

Phagocytosis of *E. coli* and RBCs

1. *E. coli* was labelled with Cy5.5 (5 mg/mL), and RBCs were labelled with NHS-biotin (20 mg/mL). Macrophages (1×10^6) were coincubated with *E. coli*-Cy5.5 or RBC- Biotin for 30 min and washed with 2% mouse serum-PBS twice. Flow cytometry analysis was carried out by using FL6 (638 nm/660 nm) to determine the phagocytosis of spleen by *E. coli.* Macrophages (coincubated with Biotin-RBCs) were incubated with streptavidin-FITC for 3 h and washed twice with 2% mouse serum PBS. FL1 (488 nm/525 nm) was used for flow cytometry analysis to determine the phagocytosis of RBCs in the spleen.

Single-cell RNA Sequencing

The spleen tissues were surgically removed and stored in MACS Tissue Storage Solution (Miltenyi Biotec, Bergisch Gladbach, Germany) until processing. Single-cell dissociation was performed by the experimentalists at the GENECHEM laboratory (Shanghai, China). Dissociated single cells were then stained for viability assessment using Calcein-AM (BD Biosciences, USA) and Draq7 (BD Biosciences). The BD Rhapsody system was used to capture transcriptomic information from single cells. Single-cell capture was achieved by random distribution of a single-cell suspension across >200,000 microwells through a limited dilution approach. Beads with oligonucleotide barcodes

were added to saturation so that a bead was paired with a cell in a microwell. The cells were lysed in the microwell to hybridize mRNA molecules to barcoded capture oligos on the beads. Beads were collected into a single tube for reverse transcription and ExoI digestion. Upon cDNA synthesis, each cDNA molecule was tagged on the 5' end (that is, the 3' end of an mRNA transcript) with a unique molecular identifier (UMI) and cell barcode indicating its cell of origin. Whole transcriptome libraries were prepared using the BD Rhapsody single-cell whole-transcriptome amplification (WTA) workflow, including random priming and extension (RPE), RPE amplification PCR and WTA index PCR. The libraries were quantified using a High Sensitivity D1000 ScreenTape (Agilent) and High Sensitivity D1000 Reagents (Agilent) on a 4150 TapeStation System (Agilent, Palo Alto, CA, USA) and the Qubit High Sensitivity DNA assay (Thermo Fisher Scientific). Sequencing was performed on an Illumina sequencer (Illumina Nova Seq 6000, San Diego, CA) in a 150 bp paired-end run.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 8.0 software. Two-tailed Student's t test or one-way analysis of variance (ANOVA) with Tukey's post hoc test. $P < 0.05$ was considered statistically significant.

Results

HH exposure promotes erythrocytosis in mice

To mimic 6000 m HA exposure, we placed C58BL/6 mice in an animal hypobaric oxygen chamber and detected the blood indices in the blood of the mice after HH exposure for different times. The blood smear showed that the number of RBCs was increased from 3 to 14 days after HH exposure compared with the normobaric normoxia (NN) group (**Figure 1A**). Routine blood tests further confirmed the results in blood smears, which showed that the RBC number (**Figure 1B**), HGB content (**Figure 1C**) and HCV value (**Figure 1D**) were all increased significantly to varying degrees, while MCH was not changed after HH exposure (**Figure 1E**). We further performed flow cytometry by TO staining to detect reticulocytes after 7 and 14 days of HH treatment. The results showed that at both HH 7 and 14 d of exposure, the reticulocyte proportion was increased (**Figure 1F**). In addition, the number of $CD47^{+}$ RBCs in the blood was also significantly increased at both 7 (**Figure 1G and H**) and 14 days (**Figure 1G and I**) after HH exposure. These results suggested that the HH-treated mouse model mimics HA exposure well, which promotes erythropoiesis and results in erythrocytosis in mice following HH exposure.

Spleen inhibits the immoderate increase in RBCs under HH conditions

To determine the roles of the spleen in RBC homeostasis under HA/HH, we investigated the effects of HH on the morphology, volume and weight of the spleen as well as erythrocyte indices. As shown in **Figure 2**, the spleen volume and weight were decreased significantly after HH exposure for 1 day compared to NN treatment (**Figure 2A-C**). However, the spleen was obviously enlarged from 2 to 14 days after HH exposure (**Figure 2A-C**). The results indicated that the spleen contracted, the stored RBCs in the spleen were released into the blood at 1 day, and the RBCs were produced and/or retained in the spleen from 2 to 14 days after HH exposure. In addition, we also investigated the effect of the spleen on RBC homeostasis under HH conditions and observed whether its clearance effect on RBCs in HH was compensated by the liver or other mononuclear macrophage systems. We removed the spleen from mice using splenectomies and then performed HH exposure for 14 days to detect the RBC counts and blood deposition. The results showed that compared with the splenectomy group mice under NN conditions or the sham group mice exposed to HH, erythrocyte deposition (**Figure 2D**) and counts (**Figure 2E**), HGB

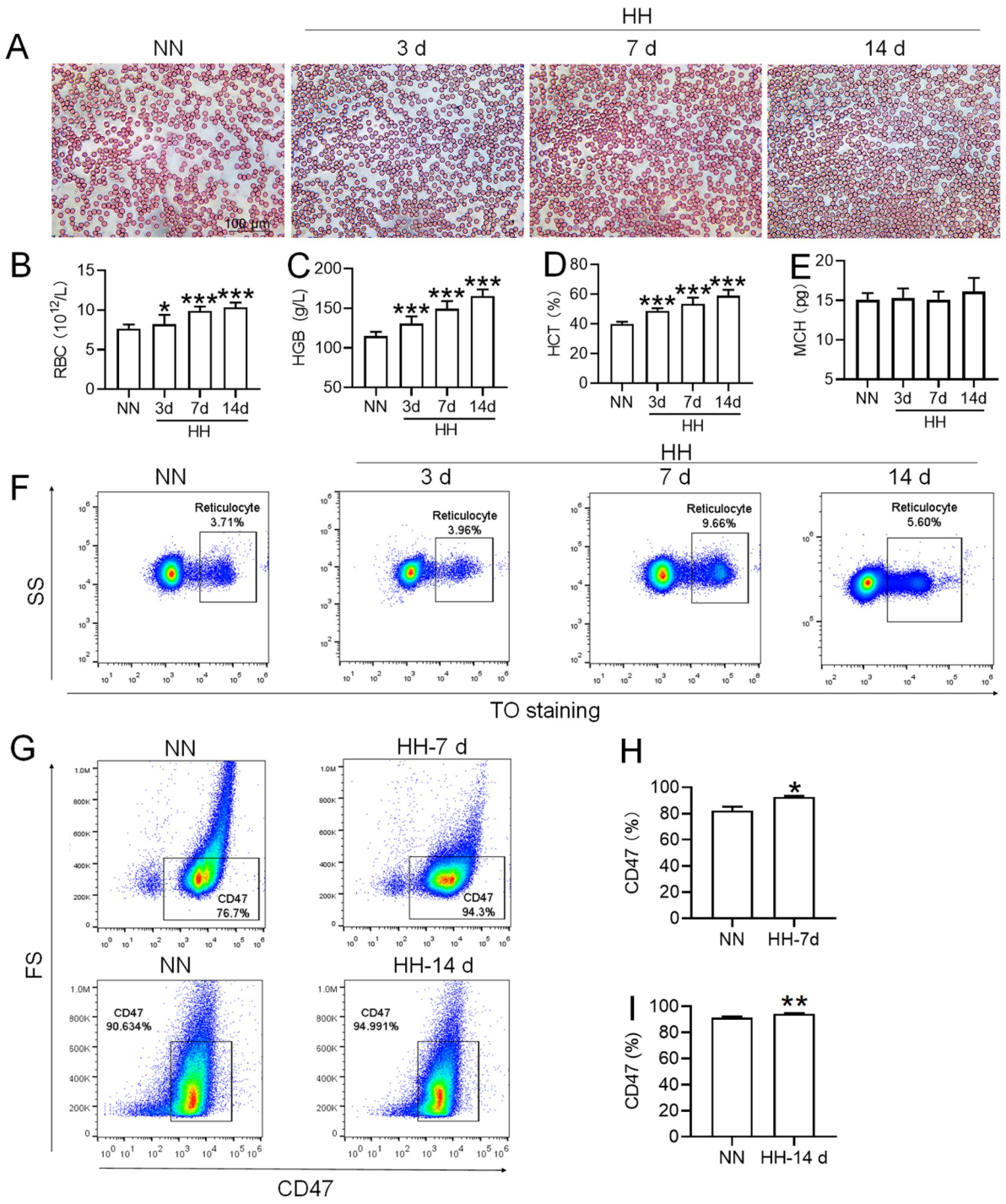

Fig. 1

HH exposure promotes erythrocytosis in mice. The C57BL/6 mice were treated with normobaric normoxia (NN) and hypobaric hypoxia (HH) for 3, 7 and 14 days, and blood was collected for the following tests. (**A**) Observation of the number and morphology of RBCs by blood cell smear. Routine blood analysis of RBC counts (**B**) and HGB (**C**), HCT (**D**), and MCH content (**E**). (**F**) Representative dot plots (one of three experiments) of TO-positive cells (reticulocytes) in total populations of spleen. (**G**) CD47 staining in splenic cells was analysed by flow cytometry. Representative dot plots (one of three experiments) of CD47 expression after HH for 7 (**H**) and 14 (**I**) days in total populations of the spleen. Data were expressed as the means ± SEM (n = 5 per group); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the NN group or the indicated group.

(**Figure 2F**) and HCV (**Figure 2G**) levels were all significantly increased 14 days after HH splenectomy, while MCH did not change (**Figure 2H**). However, these indices were not changed in the mice with or without splenectomies under NN conditions (**Figure 2D and E**). It is suggested that the liver and other mononuclear macrophages did not play an effective compensatory role under HH exposure conditions. In addition, the spleen is essential to maintain the homeostasis of RBCs after HH exposure, which can inhibit the excessive proliferation of RBCs after HH exposure.

HH exposure causes a decrease in the number of macrophages in spleen

Since macrophages are the main cell population for processing RBCs in the spleen under physiological conditions, we next investigated the counts and activity of macrophages after 7 or 14 days of HH exposure via single-cell sequencing and flow cytometry. Single-cell sequencing of the spleen showed that splenic macrophages were significantly decreased after 7 days of HH exposure (**Figure 3A-C**). Moreover, the flow cytometry of Calcein/PI double staining showed that the living cells of spleen were significantly decreased while the dead cells were significantly increased after HH exposure for 7 and 14 days (**Figure 3D-F**). We further found that both CD16 (M1 macrophage marker) and CD206 (M2 macrophage marker) expression were decreased at both 7 and 14 days of HH exposure (**Figure 3G-L**). The flow cytometry results were also consistent with the Western blot results, which showed that mature macrophages ($F4/80^+/CD11b^+$), including M1-type ($F4/80^+/CD86^+$) and M2-type ($F4/80^+/CD206^+$) macrophages, in the spleens of mice decreased after HH exposure for 14 days (**Figure 3M**). Next, to identify the migration and differentiation of monocytes from bone marrow to spleen, we further analysed the expression of chemokines CCL2, CCL7, Csf1 and Csf2 in spleen by qPCR and detected the number of monocytes in bone marrow and spleen by flow cytometry. The results showed that CCL2 and CCL7 (**Figure 3N**) and Csf1 and Csf2 (**Figure 3O**) expression were significantly decreased in the spleen after HH exposure at 7 and 14 days. Moreover, monocytes ($Ly6C^+/CD11b^+$) in the bone marrow (**Figure 3P-R**) and spleen (**Figure 3P and S**-T) also declined after HH for 7 and 14 days. Then, we assessed macrophage depletion in the spleen under HH via detection of the expression and distribution of HO-1 and F4/80. **Figure 3U** shows that both HO-1 and F4/80 were distributed in the red pulp of the spleen, and their expression was also decreased after HH exposure. These results suggested that the number of splenic macrophages decreased after HH exposure, resulting in the impairment of the processing ability of splenic erythrocytes.

HH exposure decreases erythrophagocytosis and the iron processing capacity of the spleen

We further investigated the effects of HH exposure on erythrocyte phagocytosis and heme iron recycling in splenic macrophages. We used Cy5.5 dye to label *E. coli* (**Figure 4A**) and incubated them with splenic cells from HH-exposed mice *in vitro*, and the phagocytosis of splenic cells was detected using flow cytometry (**Figure 4B**). **Figure 4B** shows that the phagocytic ability of splenic cells *in vitro* was significantly decreased after 7 days of HH exposure. In addition, we used biotin to label autologous RBCs and injected them into mice followed by HH treatment *in vivo* (**Figure 4C and D**), and the phagocytosis of splenic cells was detected using flow cytometry. At 7 and 14 days after HH, the number of biotin-labelled RBCs in blood and spleen cells decreased significantly (**Figure 4E-H**). Although the labelled RBC decay declined automatically with time, the degree of RBC decay in the HH exposure group was weaker than that in the NN group in both the blood (**Figure 4E and G**) and spleen (**Figure 4F and H**). Compared with blood, the phagocytic ability of mouse spleen macrophages to RBCs decreased more significantly after HH exposure, especially on the 14th day (**Figure 4G and H**). Furthermore, we injected Tuftsin, which can stimulate phagocytosis of macrophages, and performed immunofluorescence and iron staining to study the heme iron recycling ability of the spleen under HH conditions (**Figure 4I**).

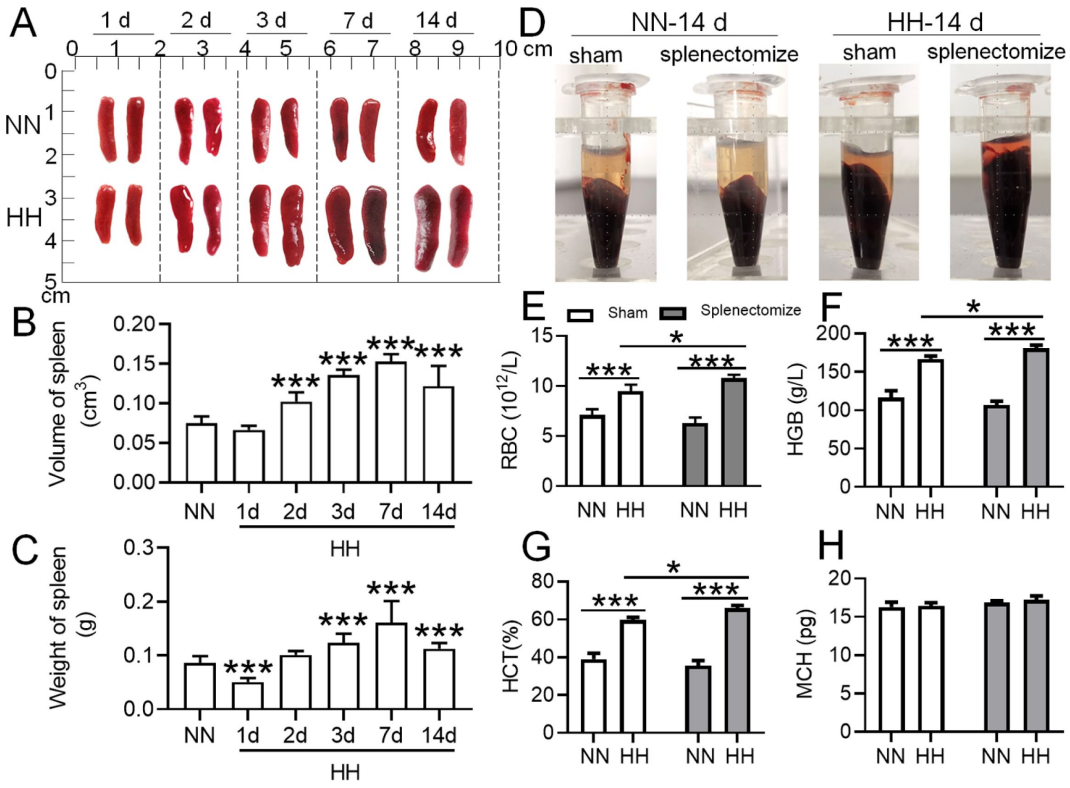

Fig. 2

The spleen plays an important role in suppressing the immoderate increase in RBCs under HH conditions. C57BL/6 mice with or without splenectomy were treated with NN and HH for different numbers of days, and the spleen and blood were collected for subsequent analyses. (**A**) Morphological observation, (**B**) spleen volume and (**C**) spleen weight detection after HH exposure. (**D-H**) Blood observation and hematological index detection. Data were expressed as the means ± SEM (n = 9 per group); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the NN group or the indicated group.

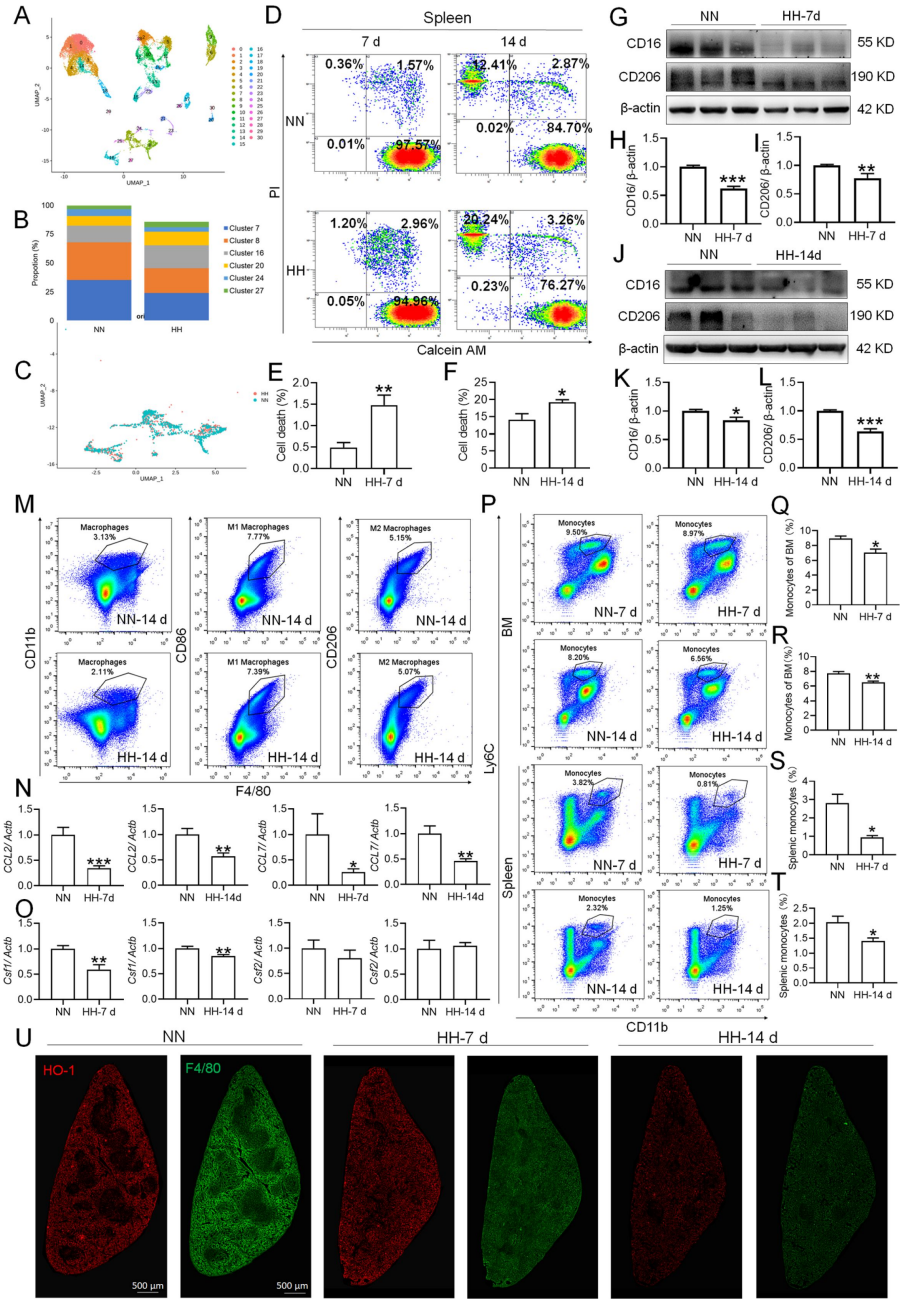

Fig. 3

HH exposure causes a decrease in the number of macrophages in spleen. The C57BL/6 mice were treated with NN and HH for 7 or 14 days, and the spleen and blood were collected for subsequent detection. (**A**) Visualization of spleen cell clusters using uniform manifold approximation and projection (UMAP). Each color represents a cluster of specific cells of the spleen, which was identified by a defined gene expression profile. (**B**) The proportions of different macrophage clusters derived from either NN-or HH-treated mouse spleens were analysed. (**C**) Individual macrophages derived from either NN-or HH-treated mouse spleens are denoted. (**D**) Calcein/PI double staining was analysed by flow cytometry. (**E and F**) Cell death proportions in total splenic cells are given as bar graphs. (**G** and **J**) Western blot detecting CD16 and CD206 protein expression in the spleen. (**H-I** and **K-L**) Statistical analysis of CD16 and CD206 expression in H and K. (**M**) F4/80 staining with CD11b, CD86 or CD206 in splenic cells was analysed by flow cytometry. (**N**) qPCR analysis of CCL2 and CCL7 expression in the spleen. (**O**) qPCR analysis of Csf1 and Csf2 expression in the spleen. (**P**) Flow cytometry detection of CD111b and Ly6C double-stained cells in the BM and spleen. $CD11b^{hi}Ly6C^{hi}$ cell proportions in the BM (**Q-R**) and spleen (**S-T**) are given as bar graphs. (**U**) HO-1 and F4/80 expression in the spleen after HH exposure for 7 and 14 days. Data were expressed as the means ± SEM (n = 3 per group); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the NN group or the indicated group.

As shown in **Figure 4J**, compared with the NN group, F4/80 expression was decreased significantly as well as Fe^{3+} deposition in the red pulp after HH exposure. However, Tuftsin induced F4/80 expression and Fe^{3+} deposition in the red pulp of the spleen under HH (**Figure 4K and L**). These results suggested that the erythrocyte phagocytosis and heme iron recycling ability of the spleen were suppressed, which was mainly due to the decrease in macrophages in red pulp under HH conditions.

HH exposure promotes iron mobilization and induces ferroptosis in the spleen

To clarify the precise mechanisms of macrophage reduction caused by HH, we examined the expression of iron metabolism-and ferroptosis-related proteins in mice treated with HH and analysed the expression of the corresponding genes in peripheral blood mononuclear cells (PBMCs) of healthy people acutely exposed to an HA environment from GEO data (No. GSE46480). The results showed that compared to the NN group, the expression levels of HO-1, Ft-L, Ft-H, NCOA4, and xCT were decreased, while the expression levels of Fpn, TfR and ACSL4 were significantly increased both at 7 and 14 days following HH exposure (**Figure 5A-B, D-E**; **Figure S1, A-D**). Except for NCOA4, the expression changes in iron metabolism and ferroptosis genes in PBMCs were consistent with our WB results (**Figure 5C and F**). There were no significant changes in the expression of the GPX4 gene and protein in human PBMCs and mouse spleens. The changes in iron metabolism-and ferroptosis-related genes in PBMCs can fully reflect the protein changes in the spleen under HH exposure. The levels of Fe^{2+} and lipid ROS in the spleen were detected by flow cytometry, and the contents of MDA, GSH and Cys were detected by biochemical detection kits. As shown in **Figure 5G and H**; Figure S1I-K, the levels of Fe^{2+} (**Figure 5G**; **Figure S1E and F**) and lipid ROS (**Figure 5H**; **Figure S1G and H**) in the spleen were significantly increased after HH exposure. In addition, the content of MDA (**Figure 5I**; **Figure S1I**) in the spleen was increased, while Cys (**Figure 5J**; **Figure S1J**) and GSH (**Figure 5K**; **Figure S1K**) were decreased significantly after HH exposure. All these results suggested that iron mobilization in the spleen was enhanced and ferroptosis was then induced after 7 days of HH exposure.

Hypoxia promotes ferroptosis of primary splenic macrophages

To study whether hypoxia exposure can induce ferroptosis in macrophages, changes in Fe^{2+} and lipid ROS levels, cell viability, phagocytosis and ferroptosis-related protein expression in primary splenic macrophages were detected under hypoxia in the presence of a ferroptosis inhibitor (Fer-1). As shown in **Figure 6**, compared with the normoxia control group (Nor), the levels of Fe^{2+} (**Figure 6A-C**) and lipid ROS (**Figure 6D-E**) were significantly increased, and the number of viable cells (**Figure 6G-H**) and phagocytosis ability (**Figure 6I**) were also significantly increased after 24 h of hypoxia (Hyp) treatment. In addition, with prolonged hypoxia time, the expression of ACSL4 gradually increased, while the expression of xCT and GPX4 decreased (**Figure 6F**). Fer-1 inhibited the increase in Fe^{2+} content (**Figure 6J-K**) and reversed the expression changes in ACSL4, xCT and GPX4 caused by hypoxia exposure (**Figure 6L-O**). At the same time, Fer-1 reversed the levels of MDA, Cys and GSH induced by hypoxia (**Figure 6P-R**). These results confirmed that hypoxia induced ferroptosis in primary splenic macrophages.

Discussion

The purpose of this study was to explore the effects and mechanisms of HA/HH exposure on erythrophagocytosis and iron circulation in mouse spleens. Here, we used an HH chamber to simulate 6000 m HA exposure and found that HH exposure induced iron mobilization and activated ferroptosis in the spleen, especially in red pulp macrophages (RPMs), which

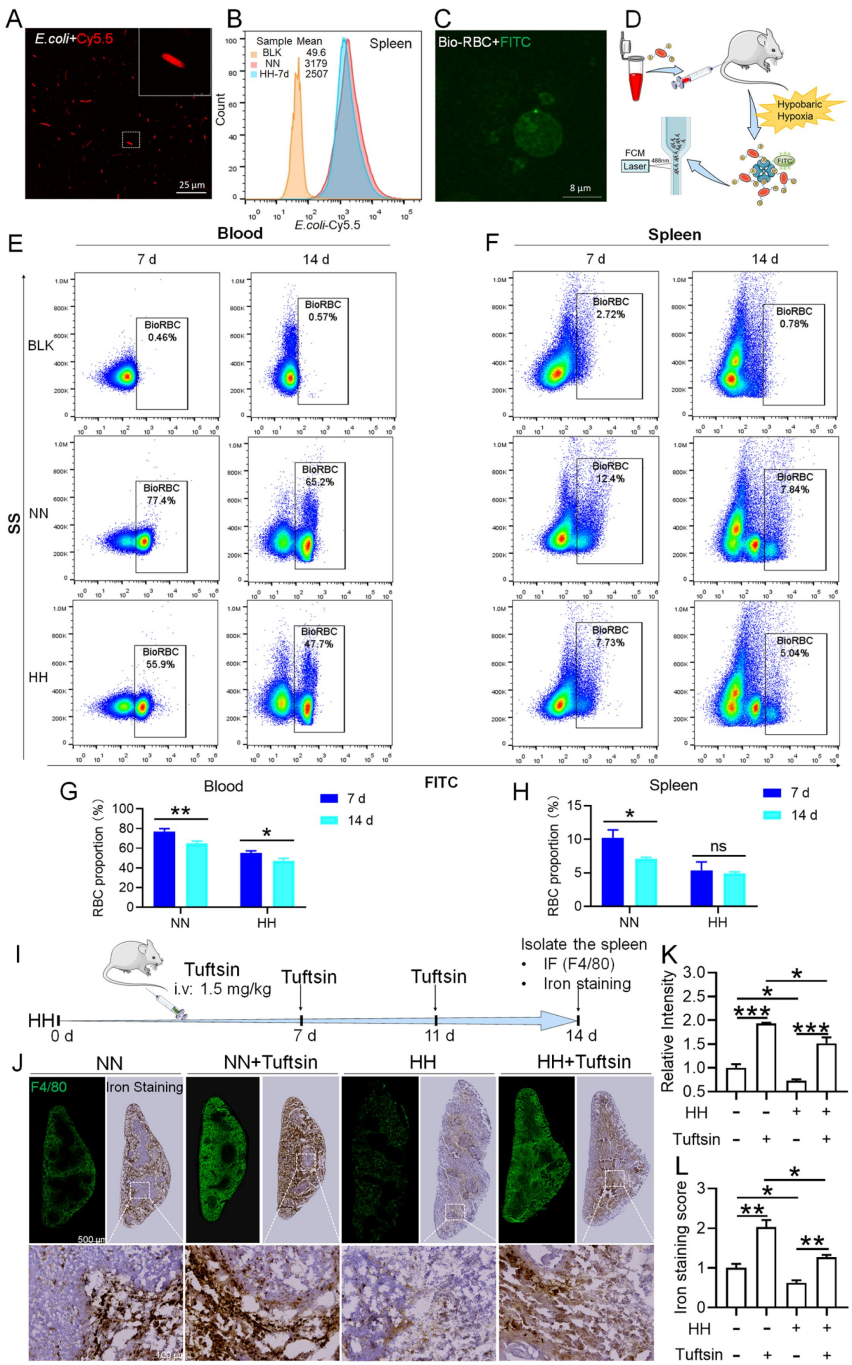

Fig. 4

HH exposure decreases erythrophagocytosis and iron processing capacity in the spleen. (**A**) *Ecoil* was labelled with Cy5.5 dye and photographed under a fluorescence microscope. (**B**) The phagocytic activity of splenic cells for Cy5.5-labelled *Ecoil* in vitro. (**C**) Autologous erythrocytes were labelled with biotin followed by NIH-FITC detection and photographed under a fluorescence microscope. (**D**) Experimental diagram of phagocytic activity in splenic cells after HH exposure in vivo. Flow cytometry detection of FITC-stained cells in the blood (**E**) and spleen (**F**) after HH exposure for 7 and 14 days. The FITC-positive cell proportions in the blood (**G**) and spleen (**H**) are given as bar graphs. (**I**) Experimental design of C57Bl6 mice (n=3 per group) treated at days 7 and 11 with a single intravenous injection of Tuftsin (1.5 mg/kg), followed by HH exposure until day 14 for isolation of the spleen for F4/80 immunohistochemistry and iron staining. (**J**) F4/80 and iron staining in the spleen after HH exposure for 14 days with Tuftsin treatment. (**K**) Quantitative results of F4/80 expression in the spleen described in (**J**). (**L**) Semiquantitative iron levels in the spleen described in (**J**). Data were expressed as the means ± SEM (n = 3 per group); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the NN group or the indicated group.

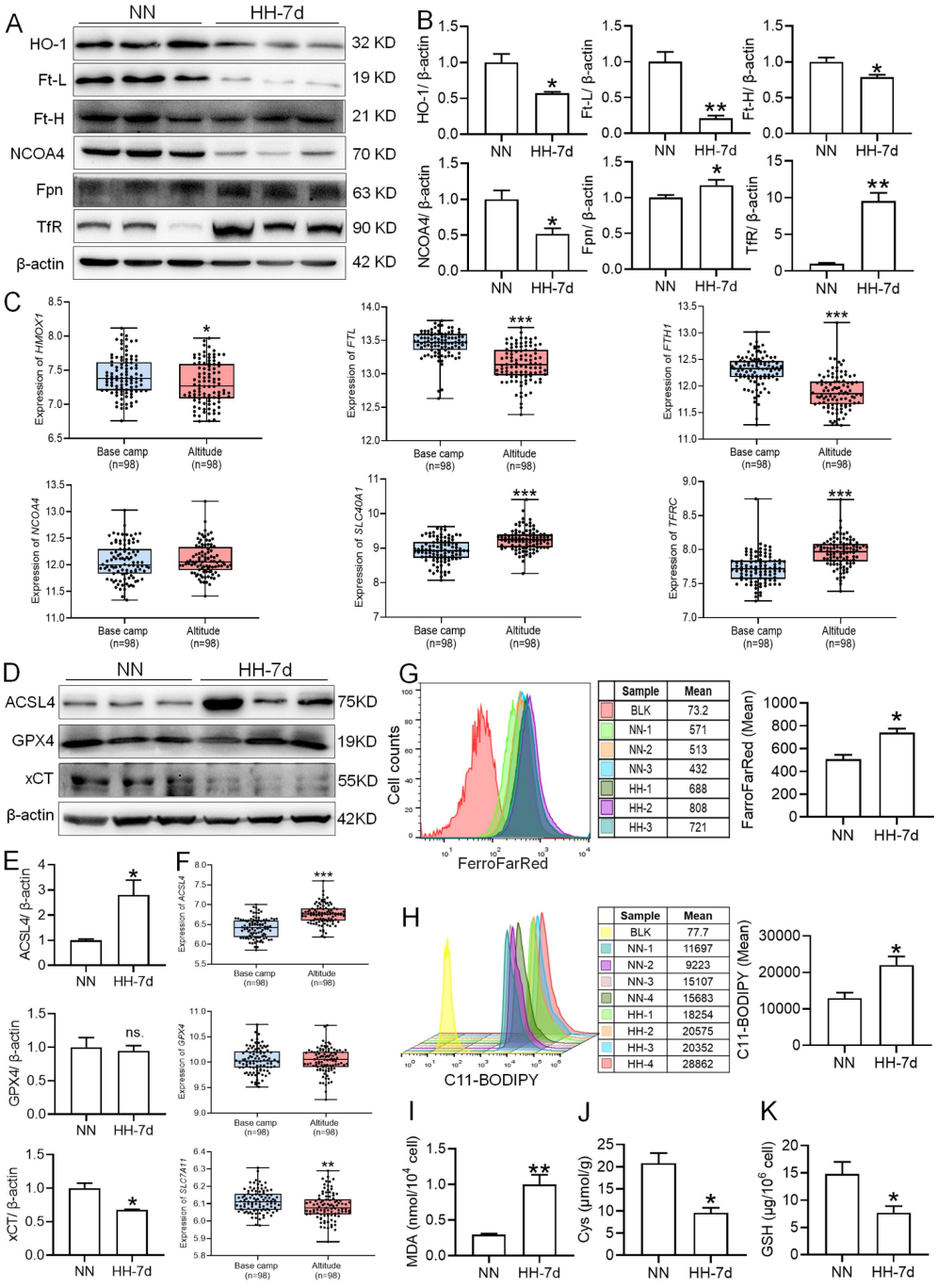

Fig. 5

HH exposure promotes iron mobilization and induces ferroptosis in the spleen. The C57BL/6 mice were treated with NN and HH for 7 days, and the spleen was collected for subsequent detection. (**A**) Western blot detection of HO-1, Ft-L, Ft-H, NCOA4, Fpn and TfR protein expression in spleen. (**B**) Statistical analysis of HO-1, Ft-L, Ft-H, NCOA4, Fpn and TfR protein expression in A. (**C**) GEO data analysis of HMOX1, FTL, FTH1, NCOA4, SLC40A1, and TFRC mRNA expression in PMBCs before and after climbing to high altitude (n=98). (**D**) Western blot detection of ACSL4, GPX4 and xCT protein expression in the spleen. (**E**) Statistical analysis of ACSL4, GPX4 and xCT protein expression in D. (**F**) GEO data analysis of ACSL4, GPX4 and SLC7A11 mRNA expression in PMBCs before and after climbing to high altitude (n=98). (**G**) The content of Fe^{2+} in the spleen was detected using the FerroFarRed probe by flow cytometry. (**H**) The level of lipid ROS in the spleen was detected using the C11-BODIPY probe by flow cytometry. The MDA (**I**), Cys (**J**) and GSH (**K**) levels in the spleen were detected using biochemical detection kits. Data were expressed as the means ± SEM (n = 3 per group); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the NN group or the indicated group.

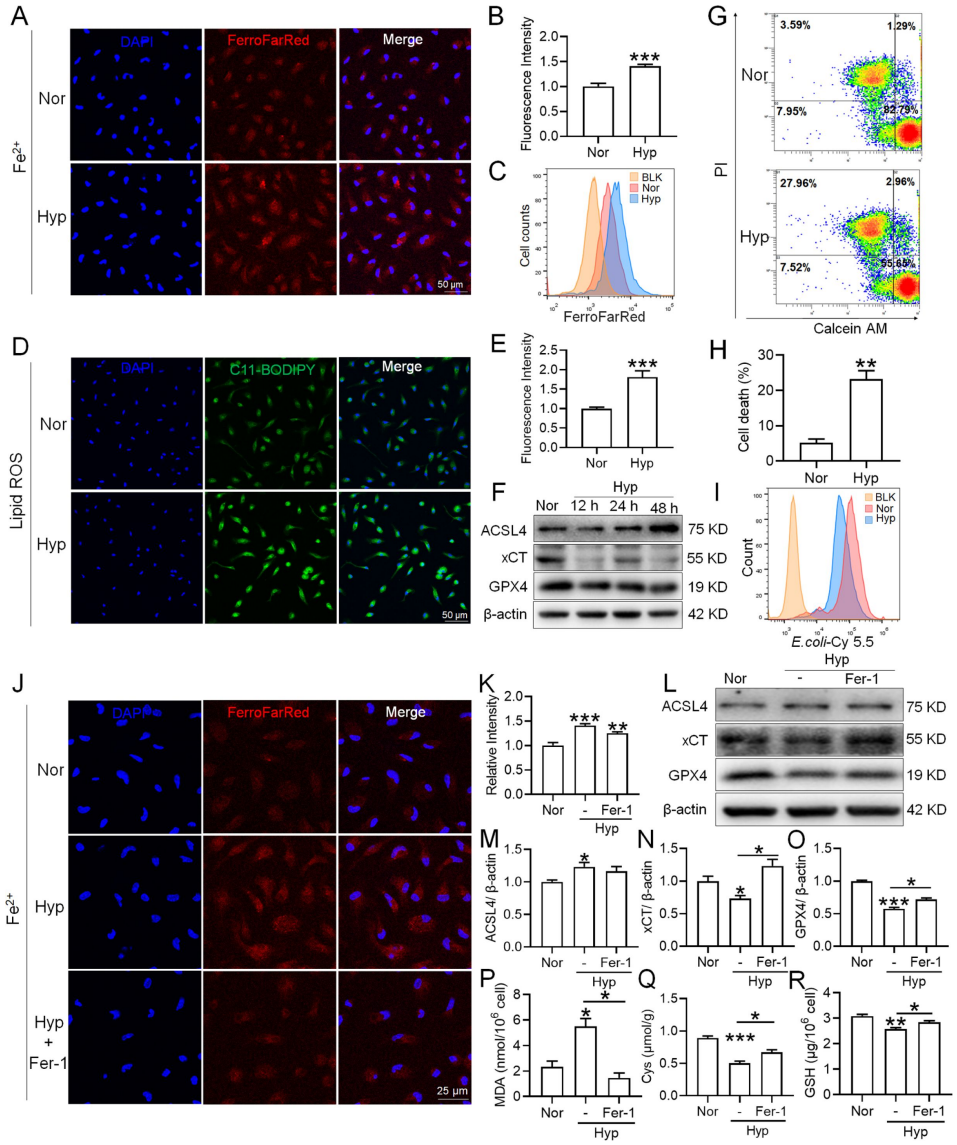

Fig. 6

Hypoxia promoted primary peritoneal macrophage ferroptosis. Primary peripheral macrophages were cultured under 1% hypoxia for different times (0, 12, 24, 48 h) with or without pre-treatment with 10 μM Fer-1 for 1 h, and then the cells were collected for subsequent detection. (**A**) Immunofluorescence detection of Fe^{2+} levels in macrophages after hypoxia exposure for 24 h using the FerroFarRed probe under a fluorescence microscope. (**B**) Quantitative results of fluorescence intensity in the macrophages described in **A**. (**C**) Flow cytometry detection of Fe^{2+} levels in macrophages exposed to hypoxia for 24 h using the FerroFarRed probe. (**D**) Immunofluorescence detection of lipid ROS levels in macrophages exposed to hypoxia for 24 h using the C11-BODIPY probe under a fluorescence microscope. (**E**) Quantitative results of mean fluorescence intensity in the macrophages described in **D**. (**F**) Western blot detection of ACSL4, GPX4 and xCT protein expression in macrophages under hypoxia for different times. (**G**) Calcein/PI double staining in macrophages after 24 h of exposure to hypoxia was analysed by flow cytometry. (**H**) Cell death proportions in macrophages are given as bar graphs. (**I**) Macrophage phagocytic activity of Cy5.5-labelled *Ecoil* after 24 h of hypoxia exposure by flow cytometry in vitro. (**J**) Immunofluorescence detection of Fe^{2+} levels in macrophages pre-treated with Fer-1 followed by 24 h of hypoxia exposure using the FerroFarRed probe. (**K**) Quantitative results of fluorescence intensity in the macrophages described in **J**. (**L**) Western blot detection of ACSL4, GPX4 and xCT protein expression in macrophages pre-treated with Fer-1 followed by 24 h of hypoxia exposure. (**M** and **N**) Statistical analysis of ACSL4, GPX4 and xCT protein expression in **L**. MDA (**P**), Cys (**Q**), and GSH (**R**) levels in macrophages pre-treated with Fer-1 followed by 24 h of hypoxia exposure were detected using kits by biochemical methods. Data were expressed as the means ± SEM (n = 3 per group); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the NN group or the indicated group.

subsequently inhibited the phagocytosis and clearance of RBCs. Finally, chronic exposure to HA/HH may promote the retention of RBCs in the spleen, cause splenomegaly, advance RBC production, and promote the occurrence and development of HAPC (**Figure 7**).

RBCs/erythrocytes are the carriers of oxygen and the most abundant cell type in the body. Erythrocytes are rapidly increased by triggering splenic contraction in acute HA/HH exposure [16] and stimulating erythropoiesis in subsequent continuous or chronic HA/HH exposure. Changes in spleen morphology and size are closely related to the spleen's ability to recover RBCs [17], and studies have shown that the spleen is in a contraction state after short-term exposure to HA/HH, and approximately 40% of the increase in RBCs is due to the contraction of the spleen [16, 18, 19]. In the present study, mice were treated under HH to mimic HA exposure (mainly mimicking the low oxygen partial pressure environment of 6000 m HA) for different times according to other studies [20, 21]. Our results showed that HH exposure not only significantly increased the RBC and HGB contents but also significantly increased $CD47^{+}$ cells in blood. However, short-term (1 d) HH exposure caused spleen contraction and induced splenomegaly after 3 d of HH treatment. These results are consistent with other research results; that is, HH or HA exposure affects spleen morphology and further affects RBC counts and HGB levels [22–24].

HAPC is a common chronic HA disease characterized by excessive proliferation of RBCs caused by HH conditions [25]. Under physiological conditions, RBC homeostasis is maintained by balancing erythropoiesis and erythrocyte clearance [4]. The ability of RBC disposal in the spleen for iron recycling under HA/HH exposure was important to RBC regeneration in bone marrow (BM) [26]. Thus, we hypothesized that erythrophagocytosis and iron recycling in the spleen were altered by HA/HH exposure and further disturbed RBC homeostasis and affected the progression of HAPC. We found that compared with sham group mice, HH significantly increased the contents of RBCs and HGB in the blood of splenectomy mice. This strongly verified that the spleen is a key organism that maintains RBC magnitude within a certain range of physiological statuses under HA/HH conditions.

As originally proposed by Metchnikoff in the 19th century, macrophages, especially RPMs, play a pivotal role in the regulation of RBCs and iron homeostasis [27, 28]. We detected the macrophage population in the spleen and found that HH exposure for 7 and 14 days reduced the total macrophage and M1-and M2-type macrophage proportions in the spleen. This result is supported by another study, that is, hypoxia reduces M1-type macrophages in human gastric cancer [29]. We next observed whether monocyte migration and differentiation from the BM to the spleen replenish reduced macrophages and sustain macrophage homeostasis after HH treatment. It is well known that splenic erythropoiesis is a dynamic process [4]. Upon phagocytosing erythrocytes in macrophages, spleen tissue (including macrophages and fibroblasts) produces the chemokine C–C motif chemokine ligand 2 and 7 (CCL2 and CCL7) to recruit blood monocytes to the spleen [30, 31]. Unlike most studies in tumours, which mainly suggested that macrophage accumulation in hypoxic areas was derived by enhanced chemokine expression in tumours [32, 33], CCL2, CCL7 and Csf1 expression was decreased in the spleen after HH exposure for 7 and 14 days in our study. Furthermore, we found that HH exposure inhibited $Ly6C^{hi}$ monocyte migration from the BM to spleen, where these cells subsequently differentiated into RPMs. The combination of decreased splenic RPMs, along with the reduced BM-derived $Ly6C^{hi}$ monocytes, suggests that the homeostasis of the RPM population of circulating monocytes was also inhibited after the RPM depletion induced by HH exposure.

HO-1 is a major antioxidant and cytoprotective enzyme that catalyzes the degradation of heme to provide heme homeostasis and protect against free heme-induced toxicity [34]. In addition, it was supposed that mice deficient in HO-1 are largely devoid of RPMs and suggested that HO-1 is critical for RPM development and survival after erythrocyte clearance [35]. The reduced HO-1 protein of red pulp in our study also implied decreased macrophage accounts induced by HH

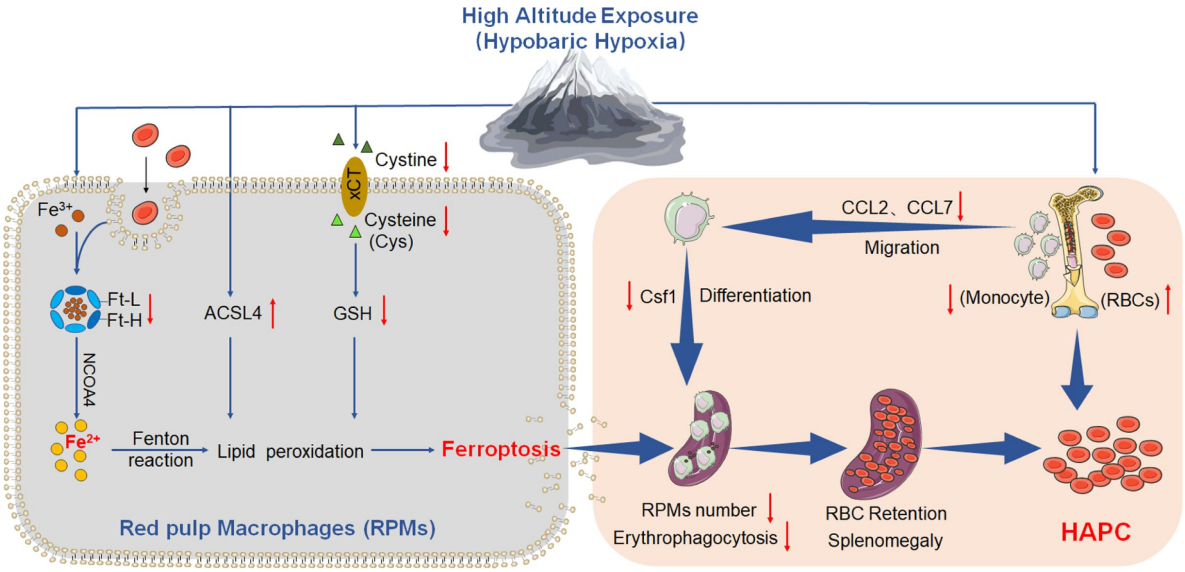

Fig. 7

High altitude/hypobaric hypoxia exposure inhibits erythrophagocytosis by inducing ferroptosis in macrophages in the spleen. HA/HH exposure induces iron mobilization, especially increased Fe^{2+} levels, which are further increased in the spleen/macrophages of mice. On the other hand, HA/HH exposure increased ACSL4 expression and inhibited GSH production by suppressing xCT expression and GSH production. The above results enhance lipid peroxidation and further macrophage ferroptosis. Meanwhile, HA/HH exposure decreases monocyte migration and differentiation from the BM to the spleen. Taken together, HA/HH exposure inhibits erythrophagocytosis and further promotes erythrocytosis, which may aggravate HAPC development.

exposure, although HO-1 was considered to be upregulated by hypoxia-inducible factor 1 (HIF-1) under hypoxia [36, 37]. Along with the decreased macrophage amount induced by HH, the phagocytic ability of macrophages in the spleen was also inhibited both *in vitro* and *in vivo*. Meanwhile, the injection of tufftsin strongly confirmed the enhancement of heme iron processing in the spleen under HH exposure. Nevertheless, our data conflict with the study of Anand *et al*, which reported that hypoxia causes an increase in phagocytosis by macrophages in a HIF-1α-dependent manner [38]. This may be because they performed intermittent hypoxia, while we used consistent hypoxia in the present study. The central role played by macrophages in supporting erythrocyte homeostasis stems, in part, from digesting the HGB content of clearance RBCs and recycling the iron back to erythroid progenitors for heme synthesis and HGB production [39]. In mammals, the majority of the body iron is in the form of heme, and the largest pool of heme consists of HGB [40]. HGB contains four prosthetic hemoglobins, and each mature RBC is thought to contain approximately 1.2×10^9 heme moieties [39]. As previously mentioned, the majority of iron required to sustain erythropoiesis is derived from recycled RBCs. In general, RPMs are equipped with molecular machinery capable of neutralizing the toxic effects of heme and metabolizing iron [41, 42]. Nevertheless, defects in erythrocytophagy in macrophages probably lead to aberrant iron metabolism, including anemia and iron overload [41]. The release of heme upon processed RBCs constitutes a permanent and considerable threat for iron cytotoxicity in macrophages and may eventually result in a specific form of programmed cell death termed ferroptosis [43], which may cause decreased cell counts [44, 45].

Accordingly, we investigated the iron metabolism status and explored ferroptosis in spleen/macrophages after HH/hypoxia treatment and found that HH exposure prompted iron mobilization, especially in ferritinophagy and lipid peroxidation in the spleen. Interestingly, we found that except for NCOA4, all the gene expression changes in the PBMCs of humans after acute HA exposure were similar to the changes in the spleens of mice after HH exposure for 7 and 14 days. This is probably because NCOA4 in the spleen mediates iron release from Ft-L and facilitates iron reuse, while PBMCs do not possess this function *in vivo*. These results not only indicated that HH exposure induces spleen ferroptosis but also implied that the gene expression changes in PBMCs under HA/HH may reflect RBC processing functions in the spleen and further indicate the iron metabolism status in the clinic. We further found that the exact mechanism of macrophage ferroptosis induced by HH exposure was caused by increased Fe^{2+} and decreased antioxidative system expression, which finally resulted in lipid peroxidation of macrophages. It has been proposed that heme catabolism by HO-1 protects macrophages from oxidative stress [41]. The enhanced ROS and lipid peroxidation *in vivo* were also consistent with the decreased HO-1 expression in the spleen. In addition, 1% hypoxia treatment induced ferroptosis *in vitro*, which was reduced by treatment with ferrostatin-1, a ferroptosis inhibitor. However, our data were inconsistent with the study of Fuhrmann *et al*., which reported that hypoxia inhibits ferritinophagy and protects against ferroptosis [46]. We hypothesized that the enhanced iron demand for new RBC regeneration in BM caused by HH leads to an increase in NCOA4 and Ft-L protein expression in the spleen. On the other hand, hypoxia inhibited anti-ferroptosis system expression, including reduced Gpx4 expression and increased lipid ROS production. The results were supported by the group of Youssef LA *et al*, who reported that increased erythrophagocytosis induces ferroptosis in RPMs in a mouse model of transfusion [47]. However, as most studies have reported, ferroptosis is not only characterized by increased lipid ROS but also significantly shrinking mitochondria [48]. We never found shrinking mitochondria in RPMs after HH exposure (data not shown). Gao *et al*. proposed that mitochondria played a crucial role in cysteine deprivation-induced ferroptosis but not in that induced by inhibiting glutathione peroxidase-4 (GPX4), the most downstream component of the ferroptosis pathway [49]. Interestingly, in our *in vivo* study, Gpx4 expression was also not changed after HH exposure. It is still not clear whether mitochondria are involved in ferroptosis. Whether HH treatment caused mitochondrial swelling directly and the exact mechanism involved in this process still need to be further investigated.

Based on the above experiments, we first put forward the "spleen theory" of HAPC (**Figure 7**). It hypothesizes that HH exposure induced spleen ferroptosis, especially RPMs, which further inhibited erythrophagocytosis, RBC clearance, HGB processing and iron recycling. Therefore, chronic exposure to HA/HH promotes the retention of RBCs in the spleen, leading to splenomegaly and the continuous production of RBCs. These findings may be clinically relevant to the pathological conditions of HAPC progression.

Abbreviations

BM: bone marrow; Cys: Cysteine; GSH: Glutathione; Ft-L: Ferritin light chains; Fpn: Ferroportin; Ft: Ferritin; HH: Hypobaric hypoxia; HA: High-altitude; HAPC: High-altitude polycythemia; HO-1: Heme oxygenase-1; HCT: Haematocrit; HGB: Haemoglobin; HIF-1a: Hypoxia inducible factor 1 a; MCH: Mean corpuscular haemoglobin; MDA: Malondialdehyde; NN: Normobaric normoxia; NCOA4: nuclear receptor coactivator 4; PBMCs: peripheral blood mononuclear cells; RBC: Red blood cell; RPM: Red pulp macrophage; TfR1: Transferrin receptor 1; Tf: Transferrin; TfR: Transferrin receptor.

Author contributions

QQL and GHW conceived, organized, and designed the study. YQG supervised the work. WPY, MQL, JD and JL performed the experiments. WPY, GW and BL contributed to the analysis of data. QQL and GHW prepared, wrote and revised the manuscript. All authors contributed to read, and approved the submitted version.

Funding

This work was supported by Natural Science Foundation of China (Grants 32271228, 82171190, and 81873924), Open Cooperation Program from Key Laboratory of Extreme Environmental Medicine, Ministry of Education (KL2019GY011), China Postdoctoral Science Foundation (2020M673649), High-level Innovation and Entrepreneurship Talents Introduction Program of Jiangsu Province of China, and Nantong Municipal Science and Technology Project (MS12021020 and MS22021010).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All animal care and experimental protocols were carried out according to the Chinese Animal Management Rules of the Ministry of Health and were authorized by the Animal Ethics Committees of Nantong University research program protocol #S20190219-011. The results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

Consent for publication

Not applicable.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

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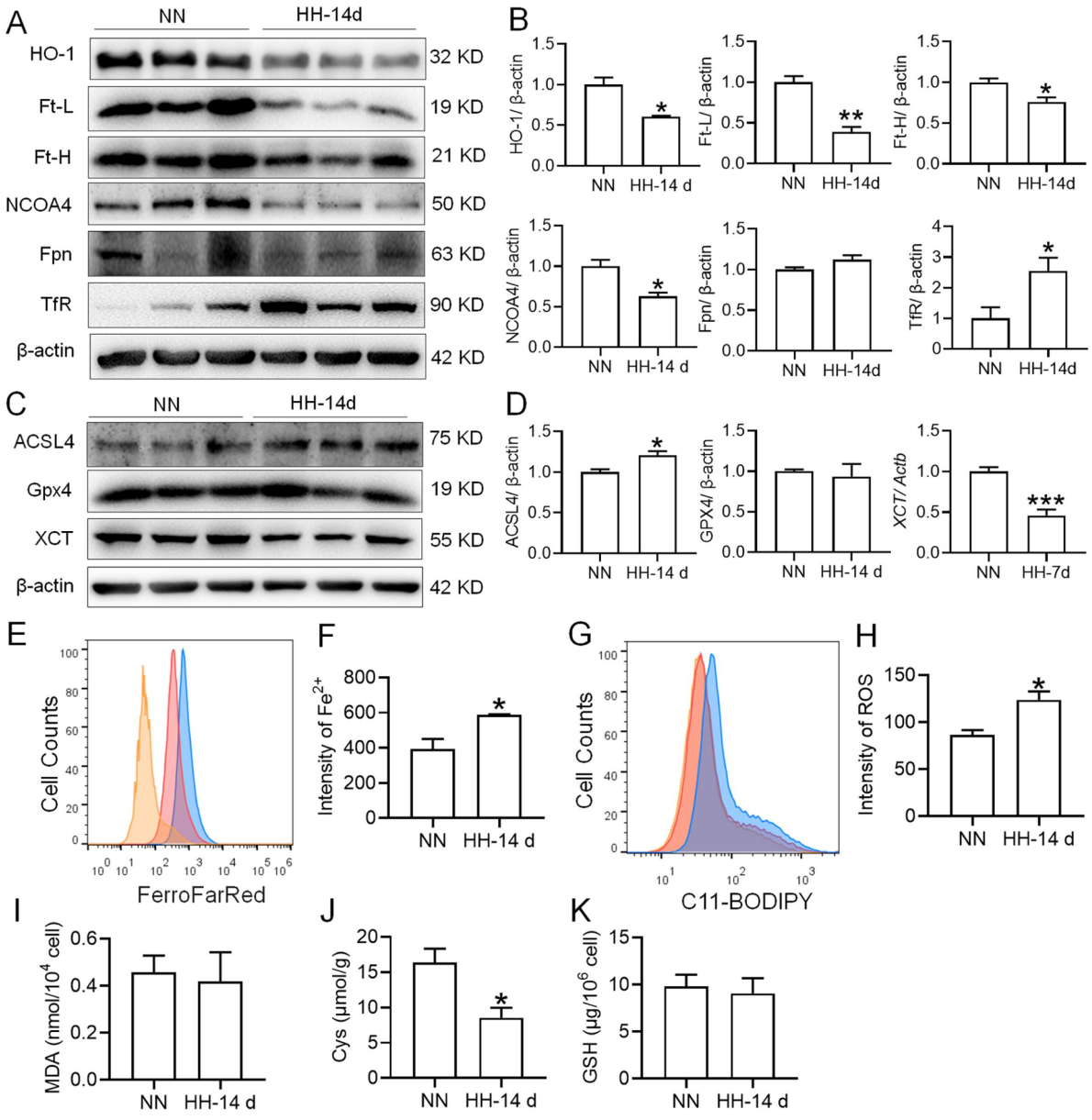

Supplementary Figure S1.

HH exposure promotes iron mobilization and induces ferroptosis in the spleen. The C57BL/6 mice were treated with NN and HH for 14 days, and the spleen was collected for subsequent detection. (**A**) Western blot detection of HO-1, Ft-L, Ft-H, NCOA4, Fpn and TfR protein expression in spleen. (**B**) Statistical analysis of HO-1, Ft-L, Ft-H, NCOA4, Fpn and TfR protein expression in **A**. (**C**) Western blot detection of ACSL4, GPX4 and xCT protein expression in the spleen. (**D**) Statistical analysis of ACSL4, GPX4 and xCT protein expression in **C**. (**E**) The content of Fe^{2+} in the spleen was detected using the FerroFarRed probe by flow cytometry. (**F**) Quantitative results of the mean fluorescence intensity in **E**. (**G**) The level of lipid ROS in the spleen was detected using the C11-BODIPY probe by flow cytometry. (**H**) Quantitative results of the mean fluorescence intensity in **G**. (**I**) MDA, (**J**) Cys, and (**K**) GSH levels in the spleen were detected using kits by biochemical methods. Data were expressed as the means ± SEM (n = 3 per group); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the NN group or the indicated group.

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Reviewer #1 (Public Review):

Wang and all present an interesting body of work focused on the effects of high altitude and hypoxia on erythropoiesis, resulting in erythrocytosis. This work is specifically focused on the spleen, identifying splenic macrophages as central cells in this effect. This is logical since these cells are involved in erythrophagocytosis and iron recycling. The results suggest that hypoxia induces splenomegaly with decreased number of splenic macrophages. There is also evidence that ferroptosis is induced in these macrophages, leading to cell destruction. Finally, the data suggest that ferroptosis in splenic red pulp macrophages causes the decrease in RBC clearance, resulting in erythrocytosis aka lengthening the RBC lifespan. However, there are many issues with the presented results, with somewhat superficial data, meaning the conclusions are overstated and there is decreased confidence that the hypotheses and observed results are directly causally related to hypoxia.

Major points:

1. The spleen is a relatively poorly understood organ but what is known about its role in erythropoiesis especially in mice is that it functions both to clear as well as to generate RBCs. The later process is termed extramedullary hematopoiesis and can occur in other bones beyond the pelvis, liver, and spleen. In mice, the spleen is the main organ of extramedullary erythropoiesis. The finding of transiently decreased spleen size prior to splenomegaly under hypoxic conditions is interesting but not well developed in the manuscript. This is a shortcoming as this is an opportunity to evaluate the immediate effect of hypoxia separately from its more chronic effect. Based just on spleen size, no conclusions can be drawn about what happens in the spleen in response to hypoxia.

2. Monocyte repopulation of tissue resident macrophages is a minor component of the process being described and it is surprising that monocytes in the bone marrow and spleen are also decreased. Can the authors conjecture why this is happening? Typically, the expectation would be that a decrease in tissue resident macrophages would be accompanied by an increase in monocyte migration into the organ in a compensatory manner.

3. Figure 3 does not definitively provide evidence that cell death is specifically occurring in splenic macrophages and the fraction of Cd11b+ cells is not changed in NN vs HH. Furthermore, the IHC of F4/80 in Fig 3U is not definitive as cells can express F4/80 more or less brightly and no negative/positive controls are shown for this panel.

4. The phagocytic function of splenic red pulp macrophages relative to infection cannot be used directly to understand erythrophagocytosis. The standard approach is to use opsonized RBCs in vitro. Furthermore, RBC survival is a standard method to assess erythrophagocytosis function. In this method, biotin is injected via tail vein directly and small blood samples are collected to measure the clearance of biotinilation by flow; kits are available to accomplish this. Because the method is standard, Fig 4D is not necessary and Fig 4E needs to be performed only in blood by sampling mice repeatedly and comparing the rate of biotin decline in HH with NN (not comparing 7 d with 14 d).

5. It is unclear whether Tuftsin has a specific effect on phagocytosis of RBCs without other potential confounding effects. Furthermore, quantifying iron in red pulp splenic macrophages requires alternative readily available more quantitative methods (e.g. sorted red pulp macrophages non-heme iron concentration).

6. In Fig 5, PBMCs are not thought to represent splenic macrophages and although of some interest, does not contribute significantly to the conclusions regarding splenic macrophages at the heart of the current work. The data is also in the wrong direction, namely providing evidence that PBMCs are relatively iron poor which is not consistent with ferroptosis which would increase cellular iron.

7. Tfr1 increase is typically correlated with cellular iron deficiency while ferroptosis consistent with iron loading. The direction of the changes in multiple elements relevant to iron trafficking is somewhat confusing and without additional evidence, there is little confidence that the authors have reached the correct conclusion. Furthermore, the results here are analyses of total spleen samples rather than specific cells in the spleen.

- https://doi.org/10.7554/eLife.87496.1.sa2

Reviewer #2 (Public Review):

The authors aimed at elucidating the development of high altitude polycythemia which affects mice and men staying in the hypoxic atmosphere at high altitude (hypobaric hypoxia; HH). HH causes increased erythropoietin production which stimulates the production of red blood cells. The authors hypothesize that increased production is only partially responsible for exaggerated red blood cell production, i.e. polycythemia, but that decreased erythrophagocytosis in the spleen contributes to high red blood cells counts.

The main strength of the study is the use of a mouse model exposed to HH in a hypobaric chamber. However, not all of the reported results are convincing due to some smaller effects which one may doubt to result in the overall increase in red blood cells as claimed by the authors. Moreover, direct proof for reduced erythrophagocytosis is compromised due to a strong spontaneous loss of labelled red blood cells, although effects of labelled E. coli phagocytosis are shown.

Their discussion addresses some of the unexpected results, such as the reduced expression of HO-1 under hypoxia but due to the above mentioned limitations much of the discussion remains hypothetical.

- https://doi.org/10.7554/eLife.87496.1.sa1

Reviewer #3 (Public Review):

The manuscript by Yang et al. investigated in mice how hypobaric hypoxia can modify the RBC clearance function of the spleen, a concept that is of interest. Via interpretation of their data, the authors proposed a model that hypoxia causes an increase in cellular iron levels, possibly in RPMs, leading to ferroptosis, and downregulates their erythrophagocytic capacity. However, most of the data is generated on total splenocytes/total spleen, and the conclusions are not always supported by the presented data. The model of the authors could be questioned by the paper by Youssef et al. (which the authors cite, but in an unclear context) that the ferroptosis in RPMs could be mediated by augmented erythrophagocytosis. As such, the loss of RPMs in vivo which is indeed clear in the histological section shown (and is a strong and interesting finding) can be not directly caused by hypoxia, but by enhanced RBC clearance. Such a possibility should be taken into account.

Major points:

1. The authors present data from total splenocytes and then relate the obtained data to RPMs, which are quantitatively a minor population in the spleen. Eg, labile iron is increased in the splenocytes upon HH, but the manuscript does not show that this occurs in the red pulp or RPMs. They also measure gene/protein expression changes in the total spleen and connect them to changes in macrophages, as indicated in the model Figure (Fig. 7). HO-1 and levels of Ferritin (L and H) can be attributed to the drop in RPMs in the spleen. Are any of these changes preserved cell-intrinsically in cultured macrophages? This should be shown to support the model (relates also to lines 487-88, where the authors again speculate that hypoxia decreases HO-1 which was not demonstrated). In the current stage, for example, we do not know if the labile iron increase in cultured cells and in the spleen in vivo upon hypoxia is the same phenomenon, and why labile iron is increased. To improve the manuscript, the authors should study specifically RPMs.

2. The paper uses flow cytometry, but how this method was applied is suboptimal: there are no gating strategies, no indication if single events were determined, and how cell viability was assessed, which are the parent populations when % of cells is shown on the graphs. How RBCs in the spleen could be analyzed without dedicated cell surface markers? A drop in splenic RPMs is presented as the key finding of the manuscript but Fig. 3M shows gating (suboptimal) for monocytes, not RPMs. RPMs are typically F4/80-high, CD11-low (again no gating strategy is shown for RPMs). Also, the authors used single-cell RNAseq to detect a drop in splenic macrophages upon HH, but they do not indicate in Fig. A-C which cluster of cells relates to macrophages. Cell clusters are not identified in these panels, hence the data is not interpretable).

3. The authors draw conclusions that are not supported by the data, some examples:

a) they cannot exclude eg the compensatory involvement of the liver in the RBCs clearance (the differences between HH sham and HH splenectomy is mild in Fig. 2 E, F and G)

b) splenomegaly is typically caused by increased extramedullary erythropoiesis, not RBC retention. Why do the authors support the second possibility? Related to this, why do the authors conclude that data in Fig. 4 G,H support the model of RBC retention? A significant drop in splenic RBCs (poorly gated) was observed at 7 days, between NN and HH groups, which could actually indicate increased RBC clearance capacity = less retention.

c) lines 452-54: there is no data for decreased phagocytosis in vivo, especially in the context of erythrophagocytosis. This should be done with stressed RBCs transfusion assays, very good examples, like from Youssef et al. or Threul et al. are available in the literature.

d) Line 475 - ferritinophagy was not shown in response to hypoxia by the manuscript, especially that NCOA4 is decreased, at least in the total spleen.

1. In a few cases, the authors show only representative dot plots or histograms, without quantification for n>1. In Fig. 4B the authors write about a significant decrease (although with n=1 no statistics could be applied here; of note, it is not clear what kind of samples were analyzed here). Another example is Fig. 6I. In this case, it is even more important as the data are conflicting the cited article and the new one: PMCID: PMC9908853 which shows that hypoxia stimulates efferocytosis. Sometimes the manuscript claim that some changes are observed, although they are not visible in representative figures (eg for M1 and M2 macrophages in Fig. 3M)

2. There are several unclear issues in methodology:

- what is the purity of primary RPMs in the culture? RPMs are quantitatively poorly represented in splenocyte single-cell suspensions. This reviewer is quite skeptical that the processing of splenocytes from approx 1 mm3 of tissue was sufficient to establish primary RPM cultures. The authors should prove that the cultured cells were indeed RPMs, not monocyte-derived macrophages or other splenic macrophage subtypes.
- (around line 183) In the description of flow cytometry, there are several missing issues. In 1) it is unclear which type of samples were analyzed. In 2) it is not clear how splenocyte cell suspension was prepared.
- In line 192: what does it mean: 'This step can be omitted from cell samples'?
- 'TO method' is not commonly used anymore and hence it was unclear to this Reviewer. Reticulocytes should be analyzed with proper gating, using cell surface markers.
- The description of 'phagocytosis of E. coli and RBCs' in the Methods section is unclear and incomplete. The Results section suggests that for the biotinylated RBCs, phagocytosis? or retention? Of RBCs was quantified in vivo, upon transfusion. However, the Methods section suggests either in vitro/ex vivo approach. It is vague what was indeed performed and how in

detail. If RBC transfusion was done, this should be properly described. Of note, biotinylation of RBCs is typically done in vivo only, being a first step in RBC lifespan assay. The such assay is missing in the manuscript. Also, it is not clear if the detection of biotinylated RBCs was performed in permeablized cells (this would be required).

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Author Response

> ***Reviewer #1 (Public Review):***
>
> *Wang and all present an interesting body of work focused on the effects of high altitude and hypoxia on erythropoiesis, resulting in erythrocytosis. This work is specifically focused on the spleen, identifying splenic macrophages as central cells in this effect. This is logical since these cells are involved in erythrophagocytosis and iron recycling. The results suggest that hypoxia induces splenomegaly with decreased number of splenic macrophages. There is also evidence that ferroptosis is induced in these macrophages, leading to cell destruction. Finally, the data suggest that ferroptosis in splenic red pulp macrophages causes the decrease in RBC clearance, resulting in erythrocytosis aka lengthening the RBC lifespan. However, there are many issues with the presented results, with somewhat superficial data, meaning the conclusions are overstated and there is decreased confidence that the hypotheses and observed results are directly causally related to hypoxia.*
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> *Major points:*
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> 1. *The spleen is a relatively poorly understood organ but what is known about its role in erythropoiesis especially in mice is that it functions both to clear as well as to generate RBCs. The later process is termed extramedullary hematopoiesis and can occur in other bones beyond the pelvis, liver, and spleen. In mice, the spleen is the main organ of extramedullary erythropoiesis. The finding of transiently decreased spleen size prior to splenomegaly under hypoxic conditions is interesting but not well developed in the manuscript. This is a shortcoming as this is an opportunity to evaluate the immediate effect of hypoxia separately from its more chronic effect. Based just on spleen size, no conclusions can be drawn about what happens in the spleen in response to hypoxia.*

Thank you for your insightful comments and questions. The spleen is instrumental in both immune response and the clearance of erythrocytes, as well as serving as a significant reservoir of blood in the body. This organ, characterized by its high perfusion rate and pliability, constricts under conditions of intense stress, such as during peak physical exertion, the diving reflex, or protracted periods of apnea. This contraction can trigger an immediate release of red blood cells (RBCs) into the bloodstream in instances of substantial blood loss or significant reduction of RBCs. Moreover, elevated oxygen consumption rates in certain animal species can be partially attributed to splenic contractions, which augment hematocrit levels and the overall volume of circulating blood, thereby enhancing venous return and oxygen delivery (Dane et al. J Appl Physiol, 2006, 101:289-97; Longhurst et al. Am J Physiol, 1986, 251: H502-9). In our investigation, we noted a significant contraction of the spleen following exposure to hypoxia for a period of one day. We hypothesized that the body, under such conditions, is incapable of generating sufficient RBCs promptly enough to facilitate enhanced oxygen delivery. Consequently, the spleen reacts by releasing its stored RBCs through splenic constriction, leading to a measurable reduction in spleen size.

However, we agree with you that further investigation is required to fully understand the implications of these changes. Considering the comments, we propose to extend our research

by incorporating more detailed examinations of spleen morphology and function during hypoxia, including the potential impact on extramedullary hematopoiesis. We anticipate that such an expanded analysis would not only help elucidate the initial response to hypoxia but also provide insights into the more chronic effects of this condition on spleen function and erythropoiesis.

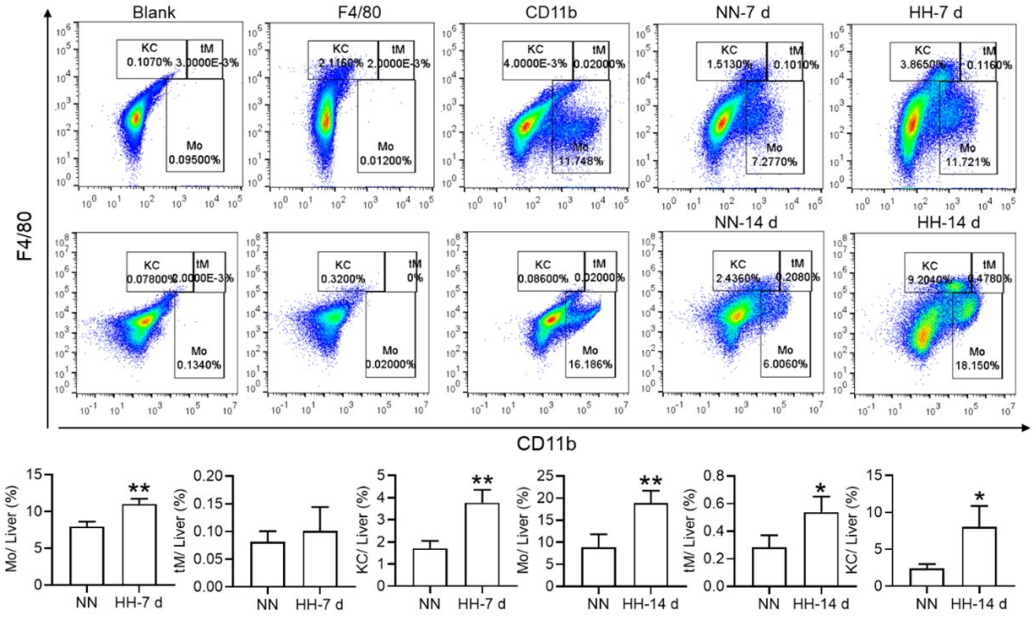
> 1. *Monocyte repopulation of tissue resident macrophages is a minor component of the process being described and it is surprising that monocytes in the bone marrow and spleen are also decreased. Can the authors conjecture why this is happening? Typically, the expectation would be that a decrease in tissue resident macrophages would be accompanied by an increase in monocyte migration into the organ in a compensatory manner.*

We appreciate your insightful query regarding the observed decrease in monocytes in the bone marrow and spleen, particularly considering the typical compensatory increase in monocyte migration into organs following a decrease in tissue resident macrophages.

The observed decrease in monocytes within the bone marrow is likely attributable to the fact that monocytes and precursor cells for red blood cells (RBCs) both originate from the same hematopoietic stem cells within the bone marrow. It is well established that exposure to hypobaric hypoxia (HH) induces erythroid differentiation specifically within the bone marrow, originating from these hematopoietic stem cells. As such, we postulate that the differentiation into monocytes is reduced under hypoxic conditions, which may subsequently cause a decrease in migration to the spleen.

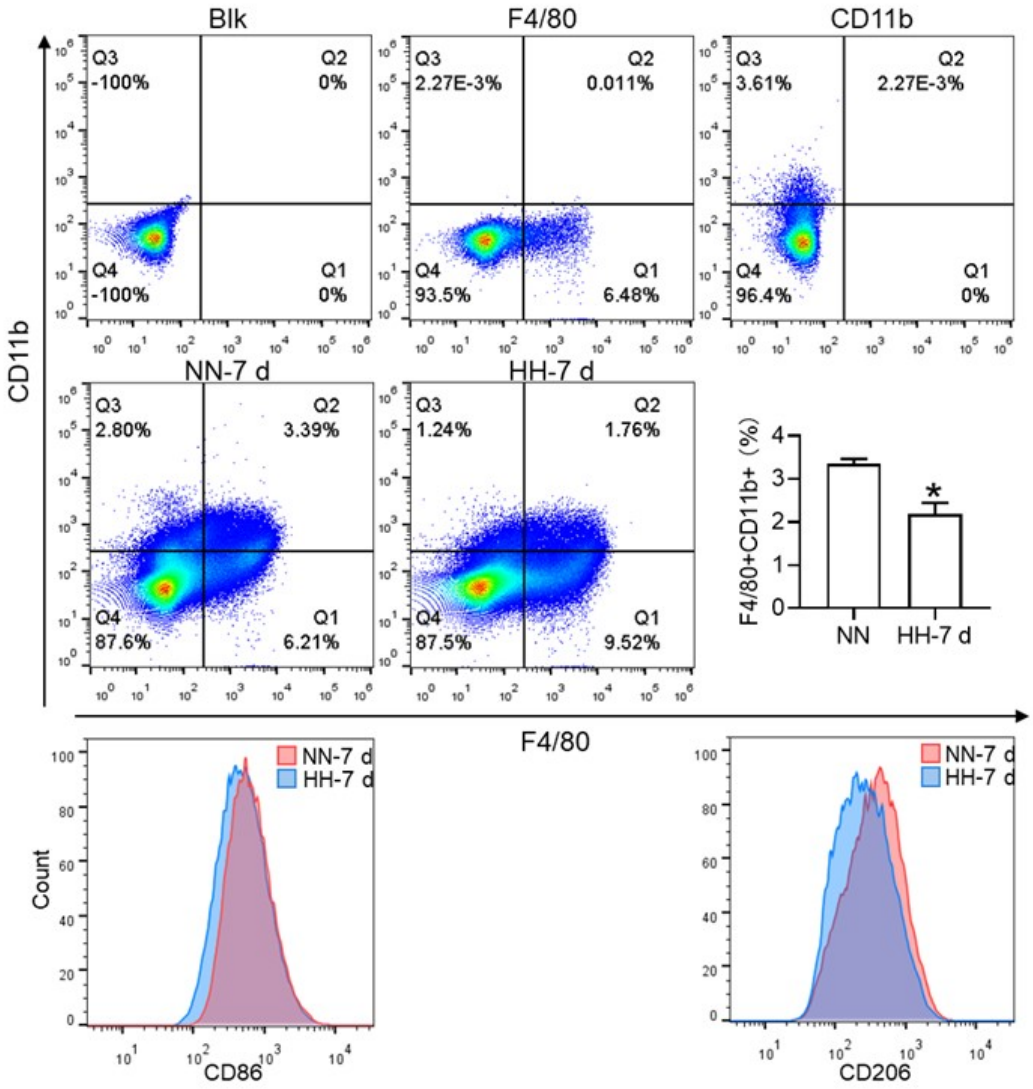
Furthermore, we hypothesize that an increased migration of monocytes to other tissues under HH exposure may also contribute to the decreased migration to the spleen. The liver, which partially contributes to the clearance of RBCs, may play a role in this process. Our investigations to date have indeed identified an increased monocyte migration to the liver. We were pleased to discover an elevation in CSF1 expression in the liver following HH exposure for both 7 and 14 days. This finding was corroborated through flow cytometry, which confirmed an increase in monocyte migration to the liver.

Consequently, we propose that under HH conditions, the liver requires an increased influx of monocytes, which in turn leads to a decrease in monocyte migration to the spleen. However, it is important to note that these findings will be discussed more comprehensively in our forthcoming publication, and as such, the data pertaining to these results have not been included in the current manuscript.

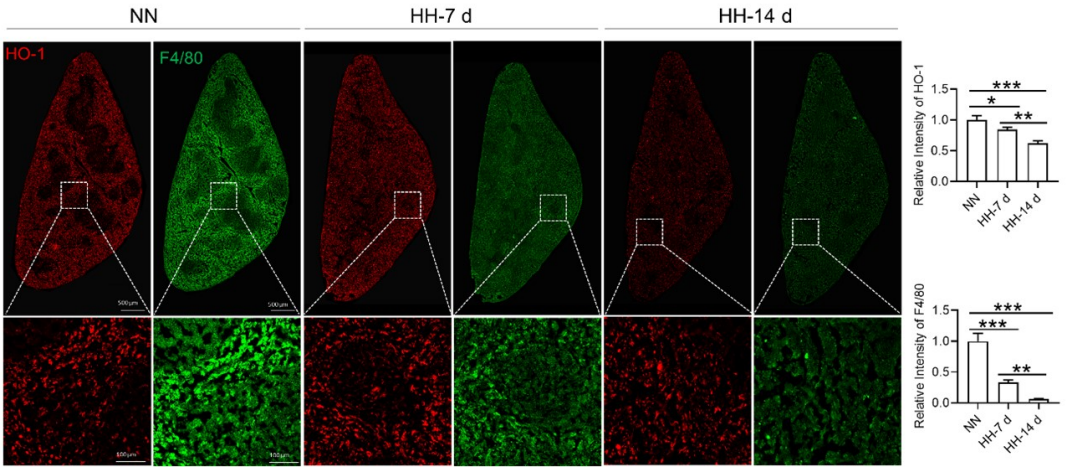


1. *Figure 3 does not definitively provide evidence that cell death is specifically occurring in splenic macrophages and the fraction of Cd11b+ cells is not changed in NN vs HH. Furthermore, the IHC of F4/80 in Fig 3U is not definitive as cells can express F4/80 more or less brightly and no negative/positive controls are shown for this panel.*

We appreciate your insightful comments and critiques regarding Figure 3. We acknowledge that the figure, as presented, does not definitively demonstrate that cell death is specifically occurring in splenic macrophages. While it is challenging to definitively determine the occurrence of cell death in macrophages based solely on Figure 3D-F, our single-cell analysis provides strong evidence that such an event occurs. We initially observed cell death within the spleen under hypobaric hypoxia (HH) conditions, and to discern the precise cell type involved, we conducted single-cell analyses. Regrettably, we did not articulate this clearly in our preliminary manuscript. In the revised version, we have modified the sequence of Figure 3A-C and Figure 3D-F for better clarity. Besides, we observed a significant decrease in the fraction of F4/80hiCD11bhi macrophages under HH conditions compared to NN. To make the changes more evident in CD86 and CD206, we have transformed these scatter plots into histograms in our revised manuscript.



Considering the limitations of F4/80 as a conclusive macrophage identifier, we have concurrently presented the immunohistochemical (IHC) analyses of heme oxygenase-1 (HO-1). Functioning as a macrophage marker, particularly in cells involved in iron metabolism, HO-1 offers additional diagnostic accuracy. Observations from both F4/80 and HO-1 staining suggested a primary localization of positively stained cells within the splenic red pulp. Following exposure to hypoxia-hyperoxia (HH) conditions, a decrease was noted in the expression of both F4/80 and HO-1. This decrease implies that HH conditions contribute to a reduction in macrophage population and impede the iron metabolism process. In the revised version of our manuscript, we have enhanced the clarity of Figure 3U to illustrate the presence of positive staining, with an emphasis on HO-1 staining, which is predominantly observed in the red pulp.



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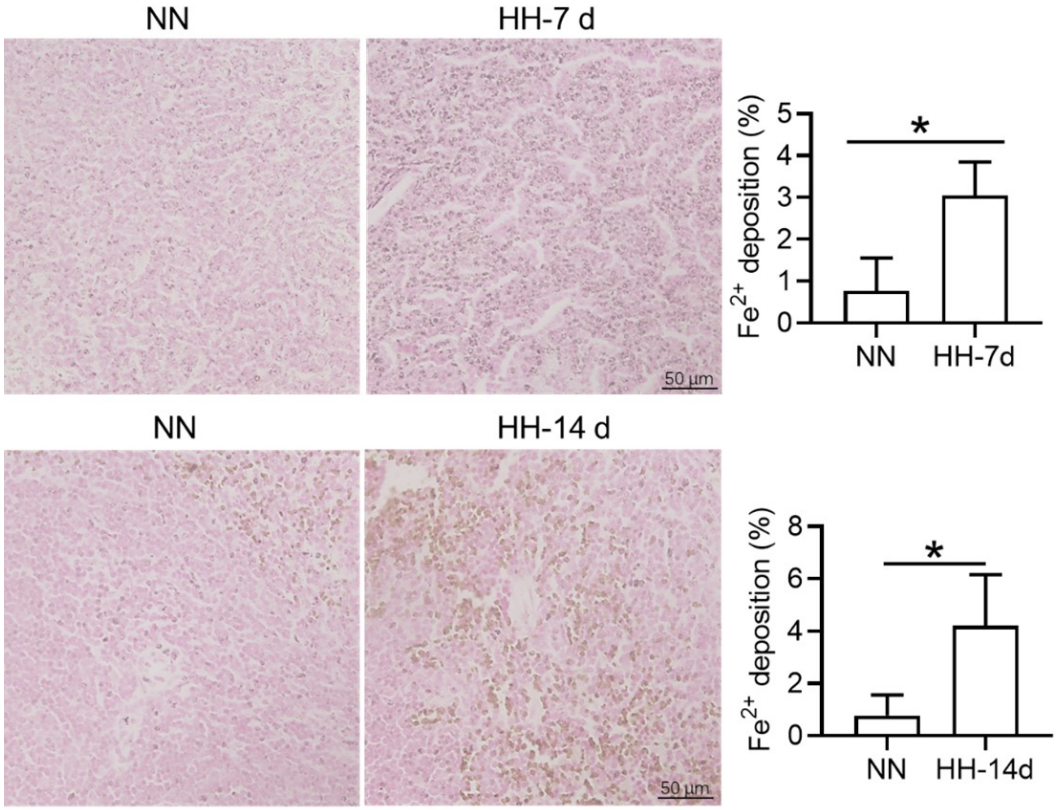
We appreciate your insightful comments and suggestions. We concur that the phagocytic function of splenic red pulp macrophages in the context of infection may not be directly translatable to understanding erythrophagocytosis. Given our assessment that the use of cy5.5-labeled E.coli alone may not be sufficient to accurately evaluate the phagocytic function of macrophages, we extended our study to include the use of NHS-biotin-labeled RBCs to assess phagocytic capabilities. While the presence of biotin-labeled RBCs in the blood could provide an indication of RBC clearance, this measure does not exclusively reflect the spleen's role in the process, as it fails to account for the clearance activities of other organs.

Consequently, we propose that the remaining biotin-labeled RBCs in the spleen may provide a more direct representation of the organ's function in RBC clearance and sequestration. Our observations of diminished erythrophagocytosis at both 7 and 14 days following exposure to HH guided our subsequent efforts to quantify biotin-labeled RBCs in both the circulatory system and spleen. These measurements were conducted during the 7 to 14-day span following the confirmation of impaired erythrophagocytosis. Comparative evaluation of RBC clearance rates under NN and HH conditions provided further evidence supporting our preliminary observations, with the data revealing a decrease in the RBC clearance rate in the context of HH conditions. In response to feedback from other reviewers, we have elected to exclude the phagocytic results and the diagram of the erythrocyte labeling assay. These amendments will be incorporated into the revised manuscript. The reviewers' constructive feedback has played a crucial role in refining the methodological precision and coherence of our investigation.

1. *It is unclear whether Tuftsin has a specific effect on phagocytosis of RBCs without other potential confounding effects. Furthermore, quantifying iron in red pulp splenic macrophages requires alternative readily available more quantitative methods (e.g. sorted red pulp macrophages non-heme iron concentration).*

We appreciate your comments and questions regarding the potential effect of Tuftsin on the phagocytosis of RBCs and the quantification of iron in red pulp splenic macrophages. Regarding the role of Tuftsin, we concur that the literature directly associating Tuftsin with erythrophagocytosis is scant. The work of Gino Roberto Corazza et al. does suggest a link between Tuftsin and general phagocytic capacity, but it does not specifically address erythrophagocytosis (Am J Gastroenterol, 1999;94:391-397). We agree that further investigations are required to elucidate the potential confounding effects and to ascertain whether Tuftsin has a specific impact on the phagocytosis of RBCs. Concerning the quantification of iron in red pulp splenic macrophages, we acknowledge your suggestion to employ readily available and more quantitative methods. We have incorporated additional Fe2+ staining in the spleen at two time points: 7 and 14 days subsequent to HH exposure (refer to the following Figure). The resultant data reveal an escalated deposition of Fe2+ within the red pulp, as evidenced in Figures 5 (panels L and M) and Figure 7 (panels L and M).



> 1. *In Fig 5, PBMCs are not thought to represent splenic macrophages and although of some interest, does not contribute significantly to the conclusions regarding splenic macrophages at the heart of the current work. The data is also in the wrong direction, namely providing evidence that PBMCs are relatively iron poor which is not consistent with ferroptosis which would increase cellular iron.*

We appreciate your insightful critique regarding Figure 5 and the interpretation of our data on peripheral blood mononuclear cells (PBMCs) in relation to splenic macrophages. We understand that PBMCs do not directly represent splenic macrophages, and we agree that any conclusions drawn from PBMCs must be considered with caution when discussing the behavior of splenic macrophages.

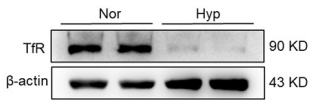
The primary rationale for incorporating PBMCs into our study was to investigate the potential correspondence between their gene expression changes and those observed in the spleen after HH exposure. This was posited as a working hypothesis for further exploration rather than a conclusive statement. The gene expression in PBMCs was congruous with changes in the spleen's gene expression, demonstrating an iron deficiency phenotype, ostensibly due to the mobilization of intracellular iron for hemoglobin synthesis. Thus, it is plausible that NCOA4 may facilitate iron mobilization through the degradation of ferritin to store iron.

It remains ambiguous whether ferroptosis was initiated in the PBMCs during our study. Ferroptosis primarily occurs as a response to an increase in Fe2+ rather than an overall increase in intracellular iron. Our preliminary proposition was that relative changes in gene expression in PBMCs could potentially mirror corresponding changes in protein expression in the spleen, thereby potentially indicating alterations in iron processing capacity post-HH exposure. However, we fully acknowledge that this is a conjecture requiring further empirical substantiation or clinical validation.

> 1. *Tfr1 increase is typically correlated with cellular iron deficiency while ferroptosis consistent with iron loading. The direction of the changes in multiple elements relevant to iron trafficking is somewhat confusing and without additional evidence, there is little confidence that the authors have reached the correct conclusion. Furthermore, the results here are analyses of total spleen samples rather than specific cells in the spleen.*

We appreciate your astute comments and agree that the observed increase in transferrin receptor (TfR) expression, typically associated with cellular iron deficiency, appears contradictory to the expected iron-loading state associated with ferroptosis. We understand that this apparent contradiction might engender some uncertainty about our conclusions.

In our investigation, we evaluated total spleen samples as opposed to distinct cell types within the spleen, a factor that could have contributed to the seemingly discordant findings. An integral element to bear in mind is the existence of immature RBCs in the spleen, particularly within the hematopoietic island where these immature RBCs cluster around nurse macrophages. These immature RBCs contain abundant TfR which was needed for iron uptake and hemoglobin synthesis. These cells, which prove challenging to eliminate via perfusion, might have played a role in the observed upregulation in TfR expression, especially in the aftermath of HH exposure. Our further research revealed that the expression of TfR in macrophages diminished following hypoxic conditions, thereby suggesting that the elevated TfR expression in tissue samples may predominantly originate from other cell types, especially immature RBCs (refer to subsequent Figure).



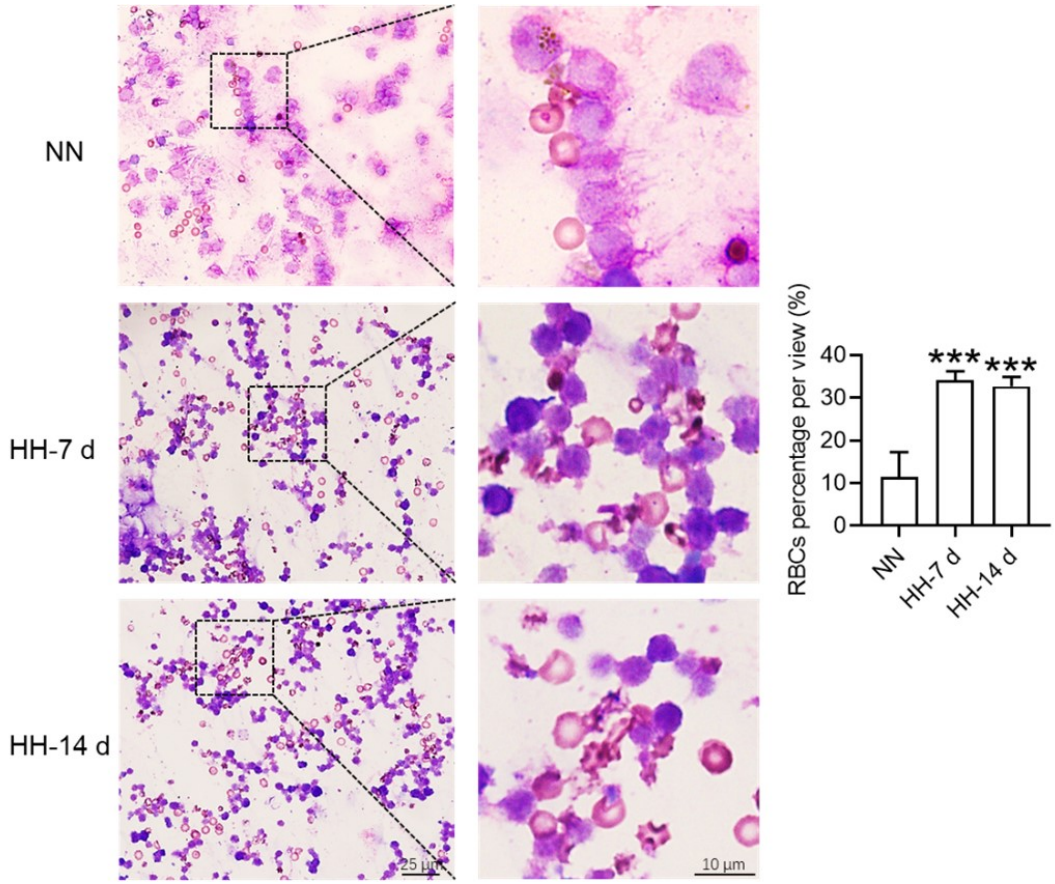
> ***Reviewer #2 (Public Review):***
>
> *The authors aimed at elucidating the development of high altitude polycythemia which affects mice and men staying in the hypoxic atmosphere at high altitude (hypobaric hypoxia; HH). HH causes increased erythropoietin production which stimulates the production of red blood cells. The authors hypothesize that increased production is only partially responsible for exaggerated red blood cell production, i.e. polycythemia, but that decreased erythrophagocytosis in the spleen contributes to high red blood cells counts.*

> *The main strength of the study is the use of a mouse model exposed to HH in a hypobaric chamber. However, not all of the reported results are convincing due to some smaller effects which one may doubt to result in the overall increase in red blood cells as claimed by the authors. Moreover, direct proof for reduced erythrophagocytosis is compromised due to a strong spontaneous loss of labelled red blood cells, although effects of labelled E. coli phagocytosis are shown. Their discussion addresses some of the unexpected results, such as the reduced expression of HO-1 under hypoxia but due to the above-mentioned limitations much of the discussion remains hypothetical.*

Thank you for your valuable feedback and insight. We appreciate the recognition of the strength of our study model, the exposure of mice to hypobaric hypoxia (HH) in a hypobaric animal chamber. We also understand your concerns about the smaller effects and their potential impact on the overall increase in red blood cells (RBCs), as well as the apparent reduced erythrophagocytosis due to the loss of labelled RBCs.

Erythropoiesis has been predominantly attributed to the amplified production of RBCs under conditions of HH. The focus of our research was to underscore the potential acceleration of hypoxia-associated polycythemia (HAPC) as a result of compromised erythrophagocytosis. Considering the spontaneous loss of labelled RBCs in vivo, we assessed the clearance rate of RBCs at the stages of 7 and 14 days within the HH environment, and subsequently compared this rate within the period from 7 to 14 days following the clear manifestation of erythrophagocytosis impairment at the two aforementioned points identified in our study. This approach was designed to negate the effects of spontaneous loss of labelled RBCs in both NN and HH conditions. Correspondingly, the results derived from blood and spleen analyses corroborated a decline in the RBC clearance rate under HH when juxtaposed with NN conditions.

Apart from the E. coli phagocytosis and the labeled RBCs experiment (this part of the results was removed in the revision), the injection of Tuftsin further substantiated the impairment of erythrophagocytosis in the HH spleen, as evidenced by the observed decrease in iron within the red pulp of the spleen post-perfusion. Furthermore, to validate our findings, we incorporated RBCs staining in splenic cells at 7 and 14 days of HH exposure, which provided concrete confirmation of impaired erythrophagocytosis (new Figure 4E).



As for the reduced expression of heme oxygenase-1 (HO-1) under hypoxia, we agree that this was an unexpected result, and we are in the process of further exploring the underlying mechanisms. It is possible that there are other regulatory pathways at play that are yet to be identified. However, we believe that by offering possible interpretations of our data and potential directions for future research, we contribute to the ongoing scientific discourse in this area.

> ***Reviewer #3 (Public Review):***
>
> *The manuscript by Yang et al. investigated in mice how hypobaric hypoxia can modify the RBC clearance function of the spleen, a concept that is of interest. Via interpretation of their data, the authors proposed a model that hypoxia causes an increase in cellular iron levels, possibly in RPMs, leading to ferroptosis, and downregulates their erythrophagocytic capacity. However, most of the data is generated on total splenocytes/total spleen, and the conclusions are not always supported by the presented data. The model of the authors could be questioned by the paper by Youssef et al. (which the authors cite, but in an unclear context) that the ferroptosis in RPMs could be mediated by augmented erythrophagocytosis. As such, the loss of RPMs in vivo which is indeed clear in the histological section shown (and is a strong and interesting finding) can be not directly caused by hypoxia, but by enhanced RBC clearance. Such a possibility should be taken into account.*

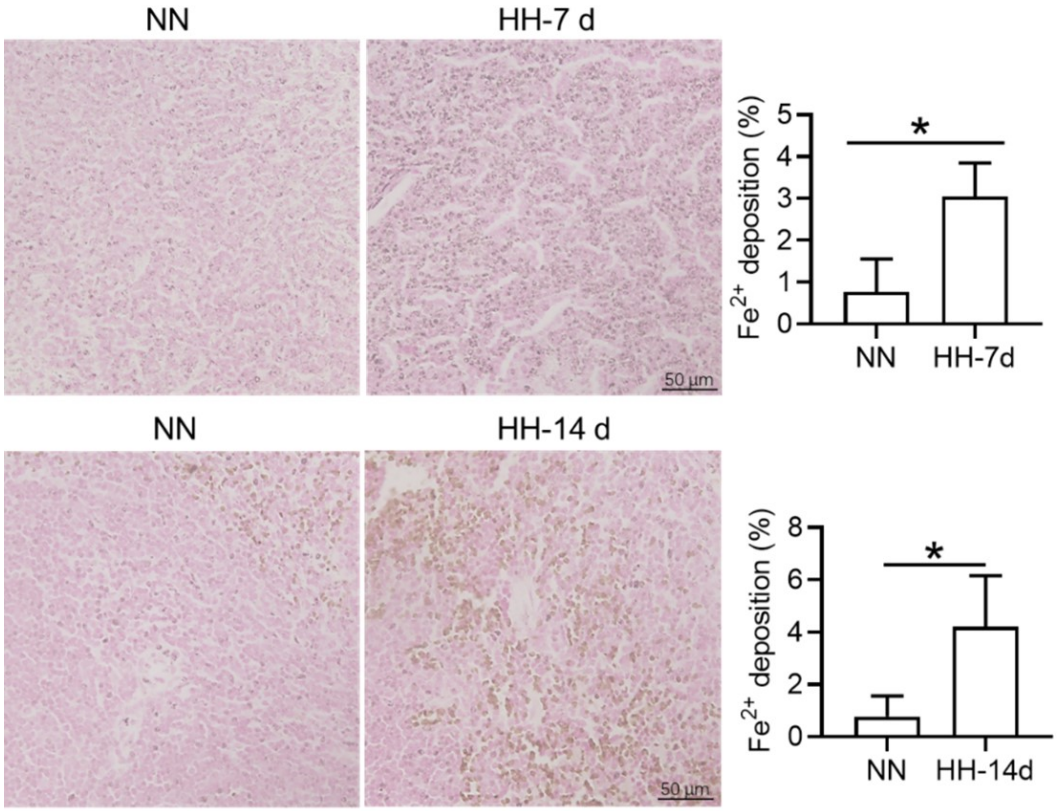
Thank you for your insightful comments and constructive feedback. In their research, Youssef et al. (2018) discerned that elevated erythrophagocytosis of stressed red blood cells (RBCs) instigates ferroptosis in red pulp macrophages (RPMs) within the spleen, as evidenced in a mouse model of transfusion. This augmentation of erythrophagocytosis was conspicuous

five hours post-injection of RBCs. Conversely, our study elucidated the decrease in erythrophagocytosis in the spleen after both 7 and 14 days.

Typically, macrophages exhibit an enhanced phagocytic capacity in the immediate aftermath of stress or stimulation. Nonetheless, the temporal points of observation in our study were considerably extended (seven and fourteen days). It remains uncertain whether phagocytic capability was amplified during the acute phase of HH exposure—particularly within the first day, considering that splenoconstriction under HH for one day results in the release of stored RBCs into the bloodstream—and whether this initial response could precipitate ferroptosis and subsequently diminished erythrophagocytosis at the 7 or 14 day marks under continued HH conditions.

> *Major points:*
>
> 1. *The authors present data from total splenocytes and then relate the obtained data to RPMs, which are quantitatively a minor population in the spleen. Eg, labile iron is increased in the splenocytes upon HH, but the manuscript does not show that this occurs in the red pulp or RPMs. They also measure gene/protein expression changes in the total spleen and connect them to changes in macrophages, as indicated in the model Figure (Fig. 7). HO-1 and levels of Ferritin (L and H) can be attributed to the drop in RPMs in the spleen. Are any of these changes preserved cell-intrinsically in cultured macrophages? This should be shown to support the model (relates also to lines 487-88, where the authors again speculate that hypoxia decreases HO-1 which was not demonstrated). In the current stage, for example, we do not know if the labile iron increase in cultured cells and in the spleen in vivo upon hypoxia is the same phenomenon, and why labile iron is increased. To improve the manuscript, the authors should study specifically RPMs.*

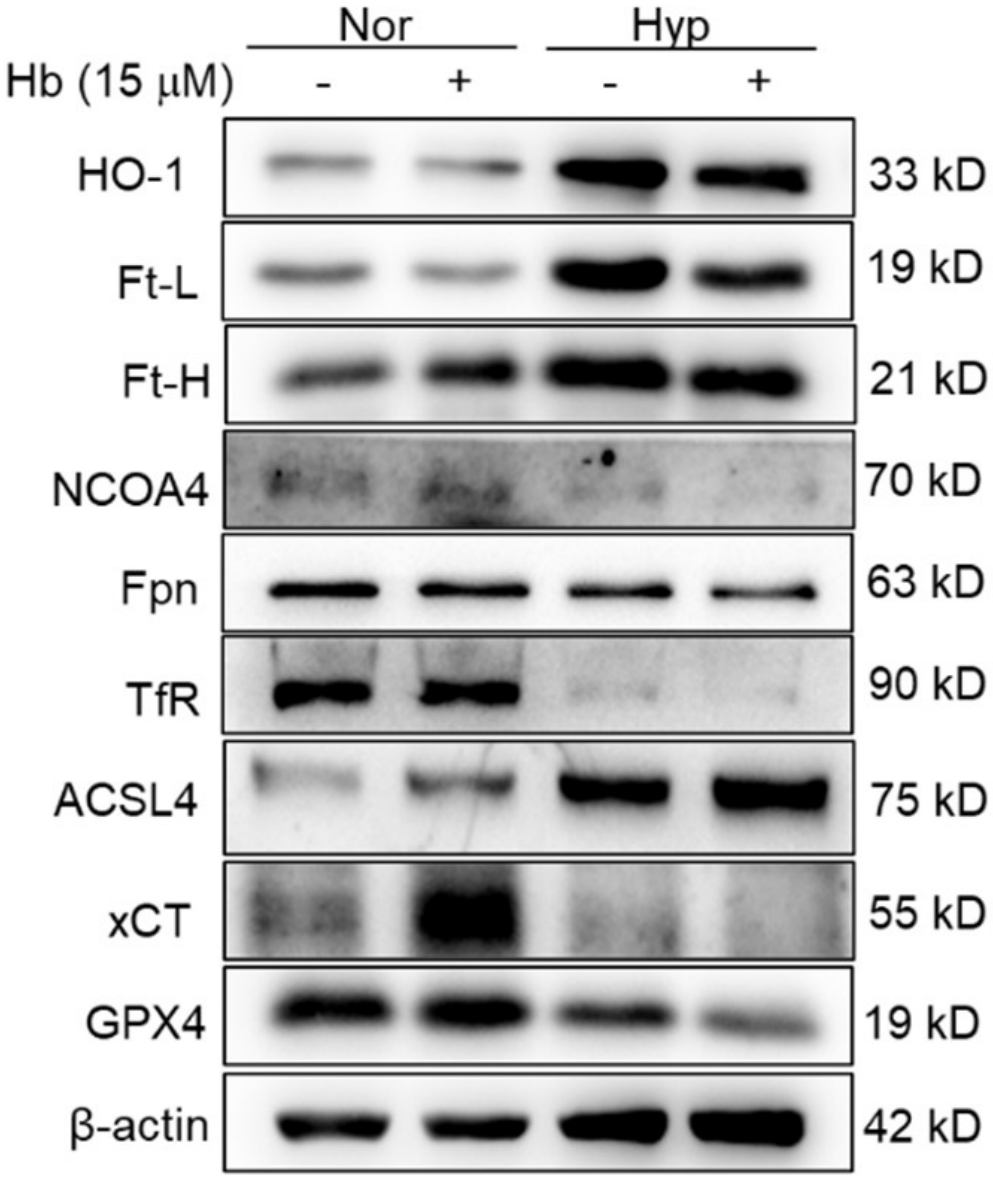
We express our gratitude for your perceptive remarks. In our initial manuscript, we did not evaluate labile iron within the red pulp and red pulp macrophages (RPMs). To address this oversight, we utilized the Lillie staining method, in accordance with the protocol outlined by Liu et al., (Chemosphere, 2021, 264(Pt 1):128413), to discern Fe^{2+} presence within these regions. The outcomes were consistent with our antecedent Western blot and flow cytometry findings in the spleen, corroborating an increment in labile iron specifically within the red pulp of the spleen.



However, we acknowledge the necessity for other supplementary experimental efforts to further validate these findings. Additionally, we scrutinized the expression of heme oxygenase-1 (HO-1) and iron-related proteins, including transferrin receptor (TfR), ferroportin (Fpn), ferritin (Ft), and nuclear receptor coactivator 4 (NCOA4) in primary macrophages subjected to 1% hypoxic conditions, both with and without hemoglobin treatment. Our results indicated that the expression of ferroptosis-related proteins was consistent with in vivo studies, however the expression of iron related proteins was not similar in vitro and in vivo. It suggesting that the increase in labile iron in cultured cells and the spleen in vivo upon hypoxia are not identical phenomena. However, the precise mechanism remains elusive.

In our study, we observed a decrease in HO-1 protein expression following 7 and 14 days of HH exposure, as shown in Figure 3U, 5A, and S1A. This finding contradicts previous research that identified HO-1 as a hypoxia-inducible factor (HIF) target under hypoxic conditions (P J Lee et al., 1997). Our discussion, therefore, addressed the potential discrepancy in HO-1 expression under HH. According to our findings, HO-1 regulation under HH appears to be predominantly influenced by macrophage numbers and the RBCs to be processed in the spleen or macrophages, rather than by hypoxia alone.

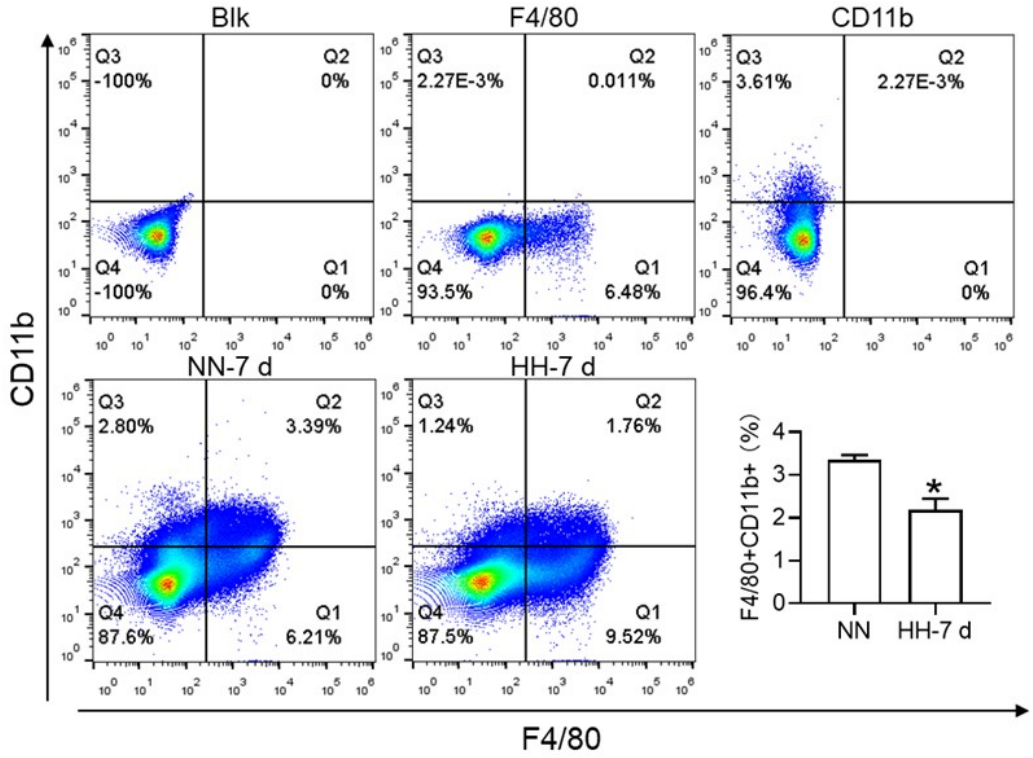
It is challenging to discern whether the increased labile iron observed in vitro accurately reflects the in vivo phenomenon, as replicating the iron requirements for RBCs production induced by HH in vitro is inherently difficult. However, by integrating our in vivo and in vitro studies, we determined that the elevated Fe2+ levels were not dependent on HO-1 protein expression, as HO-1 levels was increased in vitro while decreasing in vivo under hypoxic/HH exposure.



> 1. *The paper uses flow cytometry, but how this method was applied is suboptimal: there are no gating strategies, no indication if single events were determined, and how cell viability was assessed, which are the parent populations when % of cells is shown on the graphs. How RBCs in the spleen could be analyzed without dedicated cell surface markers? A drop in splenic RPMs is presented as the key finding of the manuscript but Fig. 3M shows gating (suboptimal) for monocytes, not RPMs. RPMs are typically F4/80-high, CD11-low (again no gating strategy is shown for RPMs). Also, the authors used single-cell RNAseq to detect a drop in splenic macrophages upon HH, but they do not indicate in Fig. A-C which cluster of cells relates to macrophages. Cell clusters are not identified in these panels, hence the data is not interpretable).*

Thank you for your comments and constructive critique regarding our flow cytometry methodology and presentation. We understand the need for greater transparency and detailed explanation of our procedures, and we acknowledge that the lack of gating strategies and other pertinent information in our initial manuscript may have affected the clarity of our findings.

In our initial report, we provided an overview of the decline in migrated macrophages (F4/80hiCD11bhi), including both M1 and M2 expression in migrated macrophages, as illustrated in Figure 3, but did not specifically address the changes in red pulp macrophages (RPMs). Based on previous results, it is difficult to identify CD11b- and CD11blo cells. We will repeat the results and attempt to identify F4/80hiCD11blo cells in the revised manuscript. The results of the reanalysis are now included (Figure 3M). However, single-cell in vivo analysis studies may more accurately identify specific cell types that decrease after exposure to HH.



Furthermore, we substantiated the reduction in red pulp, as evidenced by Figure 4J, given that iron processing primarily occurs within the red pulp. In Figure 3, our initial objective was merely to illustrate the reduction in total macrophages in the spleen following HH exposure.

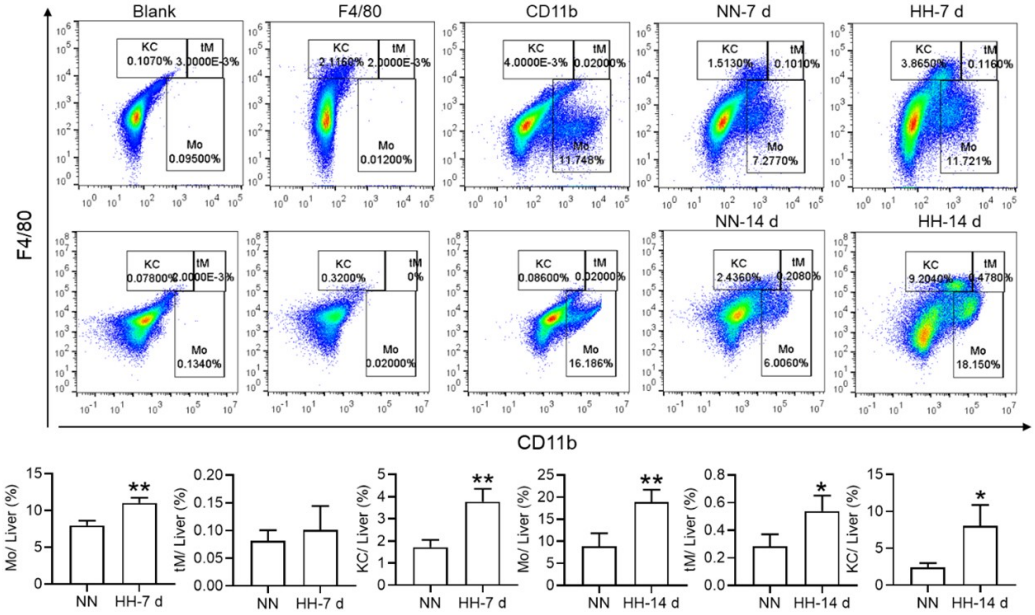
To further clarify the characterization of various cell types, we conducted a single-cell analysis. Our findings indicated that clusters 0,1,3,4,14,18, and 29 represented B cells, clusters 2, 10, 12, and 28 represented T cells, clusters 15 and 22 corresponded to NK cells, clusters 5, 11, 13, and 19 represented NKT cells, clusters 6, 9, and 24 represented cell cycle cells, clusters 26 and 17 represented plasma cells, clusters 21 and 23 represented neutrophils, cluster 30 represented erythrocytes, and clusters 7, 8, 16, 20, 24, and 27 represented dendritic cells (DCs) and macrophages, as depicted in Figure 3E.

> *1. The authors draw conclusions that are not supported by the data, some examples: a) they cannot exclude eg the compensatory involvement of the liver in the RBCs clearance (the differences between HH sham and HH splenectomy is mild in Fig. 2 E, F and G).*

Thank you for your insightful comments and for pointing out the potential involvement of other organs, such as the liver, in the RBC clearance under HH conditions. We concur with your observation that the differences between the HH sham and HH splenectomy conditions

in Fig. 2 E, F, and G are modest. This could indeed suggest a compensatory role of other organs in RBC clearance when splenectomy is performed. Our intent, however, was to underscore the primary role of the spleen in this process under HH exposure.

In fact, after our initial investigations, we conducted a more extensive study examining the role of the liver in RBC clearance under HH conditions. Our findings, as illustrated in the figures submitted with this response, indeed support a compensatory role for the liver. Specifically, we observed an increase in macrophage numbers and phagocytic activity in the liver under HH conditions. Although the differences in RBC count between the HH sham and HH splenectomy conditions may seem minor, it is essential to consider the unit of this measurement, which is value*1012/ml. Even a small numerical difference can represent a significant biological variation at this scale.

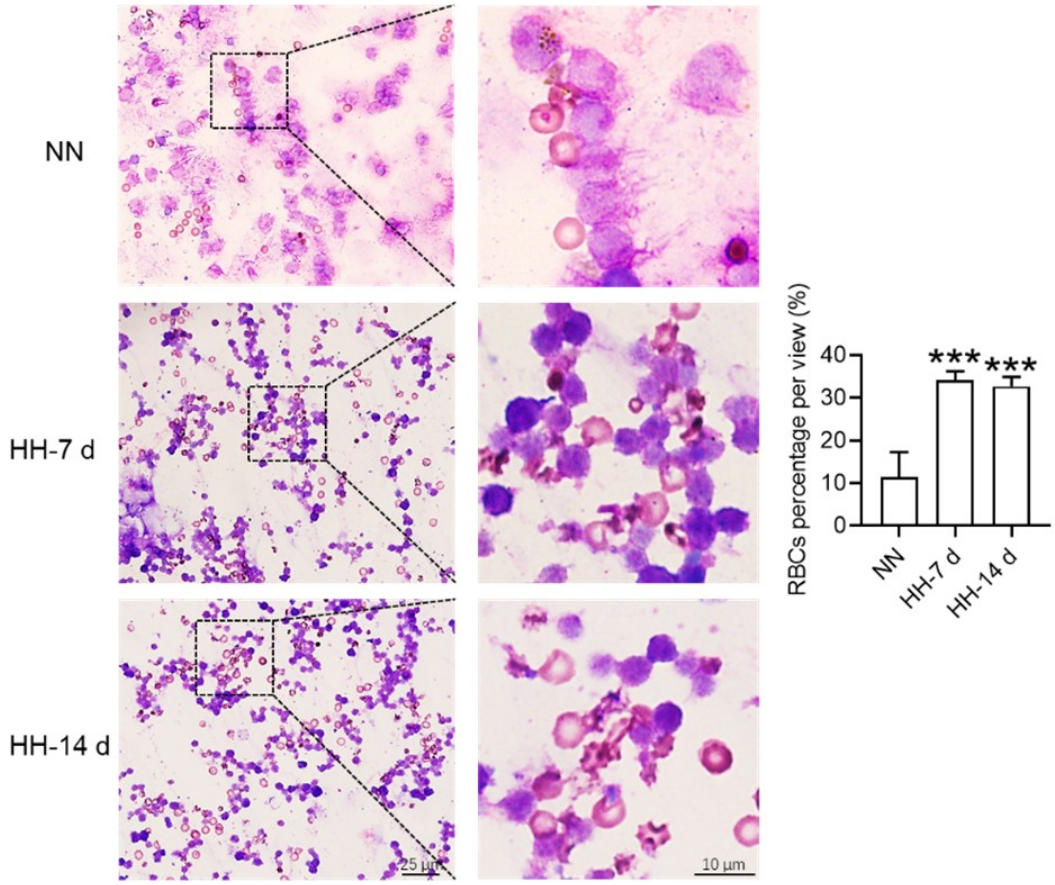


> *b) splenomegaly is typically caused by increased extramedullary erythropoiesis, not RBC retention. Why do the authors support the second possibility? Related to this, why do the authors conclude that data in Fig. 4 G,H support the model of RBC retention? A significant drop in splenic RBCs (poorly gated) was observed at 7 days, between NN and HH groups, which could actually indicate increased RBC clearance capacity = less retention.*

Prior investigations have predominantly suggested that spleen enlargement under hypoxic conditions stems from the spleen's extramedullary hematopoiesis. Nevertheless, an intriguing study conducted in 1994 by the General Hospital of Xizang Military Region reported substantial exaggeration and congestion of splenic sinuses in high altitude polycythemia (HAPC) patients. This finding was based on the dissection of spleens from 12 patients with HAPC (Zou Xunda, et al., Southwest Defense Medicine, 1994;5:294-296). Moreover, a recent study indicated that extramedullary erythropoiesis reaches its zenith between 3 to 7 days (Wang H et al., 2021).

Considering these findings, the present study postulates that hypoxia-induced inhibition of erythrophagocytosis may lead to RBC retention. However, we acknowledge that the manuscript in its current preprint form does not offer conclusive evidence to substantiate this hypothesis. To bridge this gap, we further conducted experiments where the spleen was

perfused, and total cells were collected post HH exposure. These cells were then smeared onto slides and subjected to Wright staining. Our results unequivocally demonstrate an evident increase in deformation and retention of RBCs in the spleen following 7 and 14 days of HH exposure. This finding strengthens our initial hypothesis and contributes a novel perspective to the understanding of splenic responses under hypoxic conditions.



> *c) lines 452-54: there is no data for decreased phagocytosis in vivo, especially in the context of erythrophagocytosis. This should be done with stressed RBCs transfusion assays, very good examples, like from Youssef et al. or Threul et al. are available in the literature.*

Thanks. In their seminal work, Youssef and colleagues demonstrated that the transfusion of stressed RBCs triggers erythrophagocytosis and subsequently incites ferroptosis in red pulp macrophages (RPMs) within a span of five hours. Given these observations, the applicability of this model to evaluate macrophage phagocytosis in the spleen or RPMs under HH conditions may be limited, as HH has already induced erythropoiesis in vivo. In addition, it was unclear whether the membrane characteristics of stress induced RBCs were similar to those of HH induced RBCs, as this is an important signal for in vivo phagocytosis. The ambiguity arises from the fact that we currently lack sufficient knowledge to discern whether the changes in phagocytosis are instigated by the presence of stressed RBCs or by changes of macrophages induced by HH in vivo. Nonetheless, we appreciate the potential value of this approach and intend to explore its utility in our future investigations. The prospect of distinguishing the effects of stressed RBCs from those of HH on macrophage phagocytosis is an intriguing line of inquiry that could yield significant insights into the mechanisms governing these physiological processes. We will investigate this issue in our further study.

> *d) Line 475 - ferritinophagy was not shown in response to hypoxia by the manuscript, especially that NCOA4 is decreased, at least in the total spleen.*

Drawing on the research published in eLife in 2015, it was unequivocally established that ferritinophagy, facilitated by Nuclear Receptor Coactivator 4 (NCOA4), is indispensable for erythropoiesis. This process is modulated by iron-dependent HECT and RLD domain containing E3 ubiquitin protein ligase 2 (HERC2)-mediated proteolysis (Joseph D Mancias et al., eLife. 2015; 4: e10308). As is widely recognized, NCOA4 plays a critical role in directing ferritin (Ft) to the lysosome, where both NCOA4 and Ft undergo coordinated degradation.

In our study, we provide evidence that exposure to HH stimulates erythropoiesis (Figure 1). We propose that this, in turn, could promote ferritinophagy via NCOA4, resulting in a decrease in NCOA4 protein levels post-HH exposure. We will further increase experiments to verify this concern. This finding not only aligns with the established understanding of ferritinophagy and erythropoiesis but also adds a novel dimension to the understanding of cellular responses to hypoxic conditions.

> *1. In a few cases, the authors show only representative dot plots or histograms, without quantification for n>1. In Fig. 4B the authors write about a significant decrease (although with n=1 no statistics could be applied here; of note, it is not clear what kind of samples were analyzed here). Another example is Fig. 6I. In this case, it is even more important as the data are conflicting the cited article and the new one: PMCID: PMC9908853 which shows that hypoxia stimulates efferocytosis. Sometimes the manuscript claim that some changes are observed, although they are not visible in representative figures (eg for M1 and M2 macrophages in Fig. 3M)*

We recognize that our initial portrayal of Figure 4B was lacking in precision, given that it did not include the corresponding statistical graph. While our results demonstrated a significant reduction in the ability to phagocytose E. coli, in line with the recommendations of other reviewers, we have opted to remove the results pertaining to E. coli phagocytosis in this revision, as they primarily reflected immune function. In relation to PMC9908853, which reported metabolic adaptation facilitating enhanced macrophage efferocytosis in limited-oxygen environments, it is worth noting that the macrophages investigated in this study were derived from ER-Hoxb8 macrophage progenitors following the removal of β-estradiol. Consequently, questions arise regarding the comparability between these cultured macrophages and primary macrophages obtained fresh from the spleen post HH exposure. The characteristics and functions of these two different macrophage sources may not align precisely, and this distinction necessitates further investigation.

> *1. There are several unclear issues in methodology:*
>
> - *what is the purity of primary RPMs in the culture? RPMs are quantitatively poorly represented in splenocyte single-cell suspensions. This reviewer is quite skeptical that the processing of splenocytes from approx 1 mm3 of tissue was sufficient to establish primary RPM cultures. The authors should prove that the cultured cells were indeed RPMs, not monocyte-derived macrophages or other splenic macrophage subtypes.*

Thank you for your thoughtful comments and inquiries. Firstly, I apologize if we did not make it clear in the original manuscript. The purity of the primary RPMs in our culture was found to be approximately 40%, as identified by F4/80hiCD11blo markers using flow cytometry. We recognize that RPMs are typically underrepresented in splenocyte single-cell

suspensions, and the concern you raise about the potential for contamination by other cell types is valid.

We apologize for any ambiguities in the methodological description that may have led to misunderstandings during the review. Indeed, the entirety of the spleen is typically employed for splenic macrophage culture. The size of the spleen can vary dependent on the species and age of the animal, but in mice, it is commonly approximately 1 cm in length. The spleen is then dissected into minuscule fragments, each approximately 1 mm3 in volume, to aid in enzymatic digestion. This procedure does not merely utilize a single 1 mm3 tissue fragment for RPMs cultures. Although the isolation and culture of spleen macrophages can present considerable challenges, our method has been optimized to enhance the yield of this specific cell population.

> - *(around line 183) In the description of flow cytometry, there are several missing issues. In 1) it is unclear which type of samples were analyzed. In 2) it is not clear how splenocyte cell suspension was prepared.*

1. Whole blood was extracted from the mice and collected into an anticoagulant tube, which was then set aside for subsequent thiazole orange (TO) staining. 2) Splenic tissue was procured from the mice and subsequently processed into a single-cell suspension using a 40 μm filter. The erythrocytes within the entire sample were subsequently lysed and eliminated, and the remaining cell suspension was resuspended in phosphate-buffered saline (PBS) in preparation for ensuing analyses.

We have meticulously revised these methodological details in the corresponding section of the manuscript to ensure clarity and precision.

> - *In line 192: what does it mean: 'This step can be omitted from cell samples'?*

The methodology employed for the quantification of intracellular divalent iron content and lipid peroxidation level was executed as follows: Splenic tissue was first processed into a single cell suspension, subsequently followed by the lysis of RBCs. It should be noted that this particular stage is superfluous when dealing with isolated cell samples. Subsequently, a total of 1 × 106 cells were incubated with 100 μL of BioTracker Far-red Labile Fe2+ Dye (1 mM, Sigma, SCT037, USA) for a duration of 1 hour, or alternatively, C11-Bodipy 581/591 (10 μM, Thermo Fisher, D3861, USA) for a span of 30 minutes. Post incubation, cells were thoroughly washed twice with PBS. Flow cytometric analysis was subsequently performed, utilizing the FL6 (638 nm/660 nm) channel for the determination of intracellular divalent iron content, and the FL1 (488 nm/525 nm) channel for the quantification of the lipid peroxidation level.

> - *'TO method' is not commonly used anymore and hence it was unclear to this Reviewer. Reticulocytes should be analyzed with proper gating, using cell surface markers.*

We are appreciative of your astute observation pertaining to the methodology we employed to analyze reticulocytes in our study. We value your recommendation to utilize cell surface markers for effective gating, which indeed represents a more modern and accurate approach. However, as reticulocyte identification is not the central focus of our investigation, we opted for the TO staining method—due to its simplicity and credibility of results. In our initial exploration, we adopted the TO staining method in accordance with the protocol outlined (Sci Rep, 2018, 8(1):12793), primarily owing to its established use and demonstrated efficacy in reticulocyte identification.

- *The description of 'phagocytosis of E. coli and RBCs' in the Methods section is unclear and incomplete. The Results section suggests that for the biotinylated RBCs, phagocytosis? or retention? Of RBCs was quantified in vivo, upon transfusion. However, the Methods section suggests either in vitro/ex vivo approach. It is vague what was indeed performed and how in detail. If RBC transfusion was done, this should be properly described. Of note, biotinylation of RBCs is typically done in vivo only, being a first step in RBC lifespan assay. The such assay is missing in the manuscript. Also, it is not clear if the detection of biotinylated RBCs was performed in permeablized cells (this would be required).*

Thanks for the comments. In our initial methodology, we employed Cy5.5-labeled Escherichia coli to probe phagocytic function, albeit with the understanding that this may not constitute the most ideal model for phagocytosis detection within this context (in light of recommendations from other reviewers, we have removed the E. coli phagocytosis results from this revision, as they predominantly mirror immune function). Our fundamental aim was to ascertain whether HH compromises the erythrophagocytic potential of splenic macrophages. In pursuit of this, we subsequently analyzed the clearance of biotinylated RBCs in both the bloodstream and spleen to assess phagocytic functionality in vivo.

In the present study, instead of transfusing biotinylated RBCs into mice, we opted to inject N-Hydroxysuccinimide (NHS)-biotin into the bloodstream. NHS-biotin is capable of binding with cell membranes in vivo and can be recognized by streptavidin-fluorescein isothiocyanate (FITC) after cells are extracted from the blood or spleen in vitro. Consequently, biotin-labeled RBCs were detectable in both the blood and spleen following NHS-biotin injection for a duration of 21 days.

Ultimately, we employed flow cytometry to analyze the NHS-biotin labeled RBCs in the blood or spleen. This method facilitates the detection of live cells and is not applicable to permeabilized cells. We believe this approach better aligns with our investigative goals and offers a more robust evaluation of erythrophagocytic function under hypoxic conditions.