Macrophage-HFE controls iron metabolism and immune responses in aged mice

Naveen Kumar Tangudu,[1] Dilay Yilmaz,[1] Katharina Wörle,[2] Andreas Gruber,[2] Silvia Colucci,[3] Kerstin Leopold,[2] Martina U. Muckenthaler[3] and Maja Vujić Spasić [1]

[1]Institute of Comparative Molecular Endocrinology, Ulm University, Ulm; [2]Institute of Analytical and Bioanalytical Chemistry, Ulm University, Ulm and [3]Department of Pediatric Hematology, Oncology and Immunology, University of Heidelberg, Molecular Medicine Partnership Unit, Heidelberg, Germany

Correspondence:
MAJA VUJIĆ SPASIĆ - maja.vujic@uni-ulm.de

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Material and Methods

Animal care and experimentations

Hfe^{LysMCre}, *Hfe*$^{-/-}$ and wild-type female mice, maintained on a C57BL/6J genetic background, were used in this study at the age of 12 and 45 weeks. All mice were maintained under a constant dark-light cycle, and were allowed access to food and water *ad libitum*. Mice were kept under a standard mouse diet containing 180mg/kg iron (Ssniff, Soest, Germany). Mice were euthanized by CO_2 inhalation. Heparinized blood was obtained to assess hematological parameters, and liver, spleen, duodenum were collected and stored at –80°C. Animal experiments were approved and performed in accordance to the University Animal Care Committee and the Federal Authorities for Animal Research (Regierungspraesidium Tuebingen, Baden-Wuerttemberg, Germany).

Iron deficiency was induced by feeding mice with a low iron diet (containing 3 mg/kg or 3ppm iron, Ssniff, Soest, Germany) for 5 weeks starting of age 39-40 weeks.

Acute systemic iron overload was induced by single i.p. administration of 0.2g of iron dextran solution (100 mg/ml solution, containing 50,000 ppm iron, Sigma Aldrich, Germany) per kg weight of mouse for a week.

LPS-induced septic shock was induced by single i.p injection of LPS (10mg/kg; LPS from *E. coli* 0111:B4, L2630, Sigma Aldrich, Germany). 12h, 24h, and 36h later, blood was retrieved via tail vein and retro-orbital way into an EDTA-coated tube. Blood samples were centrifuged (15min/1000g at 4°C), the plasma was separated and stored at -80°C for analysis. Mice were not sacrificed at the time of sampling. A health monitoring system was introduced to monitor the health of animals which included measuring the body weight, the temperature and scoring animal behavior. Mice were euthanized when the body temperature dropped below 30°C, the body weight decreased by 15% and mice showed significant deterioration in their behavior including the presence of lethargy.

Isolation of primary liver cells and bone marrow-derived macrophages

Primary murine hepatocytes, Kupffer cells, liver sinusoidal endothelial cells and hepatic stellate cells were isolated from livers of 45-weeks-old *Hfe*^{LysMCre} mutant and control mice by a two-step portal vein collagenase perfusion method.

Bone marrow-derived macrophages (BMDMs) were obtained by flushing femoral marrows from *Hfe*$^{-/-}$, *Hfe*^{LysMCre} and *Hfe*^{AlfpCre} mutant mice. Cells were plated at density 1×10^6 cells/ml in culture petri dishes (Becton Dickinson, USA) using Dulbecco's Minimal Eagles Medium (DMEM; Invitrogen, USA) supplemented with 10% Fetal Bovine Serum, 10mM Sodium-Pyruvate, 10mM L-Glutamine, penicillin and streptomycin (Sigma Aldrich, St. Louis, USA). The cells were differentiated with 20% mouse L929 fibroblast cell line culture supernatant as a source for macrophage-colony stimulating factor. Following 4 days culture, non-adherent cells were removed and adherent cells, bone marrow derived macrophages (BMDMs) were washed twice with PBS and the medium was replaced daily for 7 days, when the cells were used for the experiments.

Plasma cytokine measurements

Plasma cytokine concentrations were determined using the Bio-Plex Pro Mouse Cytokine Panel 23-plex (Bio-Rad Laboratories Inc., USA) according to the manufacturer's instructions. Cytokine concentrations were calculated with BioRad Bioplex Manager 6.1 software by using a standard curve derived from a recombinant cytokine standard. Cytokine concentrations are shown as pg/ml.

Enzyme-linked immunosorbent assays (ELISA)

Hepcidin was measured in mouse sera using HAMP ELISA kit (Intrinsic Lifesciences, USA) according to the manufacturer's instructions. For mouse sera obtained from LPS-experiments,

1:120 dilutions of sera were used. Erythropoietin (EPO) was measured in mouse sera using EPO ELISA kit (R&D) according to the manufacturer's instructions.

Hematology, iron measurements and staining

Hematological indices were determined using the ABC ScilVet analyzer (ABX Diagnostics). Plasma iron, tissue non-heme iron, and Perls' stainings (used to visualise iron deposition on livers, spleens and duodenum) were performed as previously described (1).

Measurement of the intracellular total iron content using total-reflection X-ray fluorescence (TXRF)

Intracellular total iron levels in BMDMs, primary hepatocytes (HC), Kupffer cells (KC), liver sinusoidal endothelial cells (LSEC) and hepatic stellate cells (HSC) were measured by total-reflection X-ray fluorescence (TXRF). To avoid trace contamination, all materials were treated with 0.5% HNO3 for 12 hours, and rinsed 3 times with ultra-pure water (type I water, MilliQ Reference A+, Merck Millipore, Germany) prior to use – a standard procedure in trace metal analysis. In brief, more than 10^5 cells were rinsed with PBS and pelleted in pre-cleaned reaction tubes. Cell pellets were digested in 1 ml high-purity concentrated HNO_3 (sub-boiled, DST-1000 Acid Purification System, Savillex, USA) and homogenized by vigorous shaking for a few minutes. As an internal standard 10 µl of Gallium solution in 2% HNO_3 was added to each sample (Gallium standard, 1:10 diluted; AAS Standard 1000 mg/L in 2% nitric acid, AVS Titrinorm, VWR Prolabo, Belgium). Aliquots of 10 µl of these digests were then pipetted onto pre-cleaned quartz glass carriers in duplicates and dried at 60 °C. Each sample carrier was measured in triplicate using a *S2 Picofox* benchtop TXRF with a Mo X-ray tube (high-efficiency module, Bruker Nano GmbH, Germany) and a live time of 500 s. A Bayesian deconvolution was applied to the spectra (optimized fit, max. stripping cycles: 100, step width: 1).

Flow cytometry

Spleens and bone marrow from 45-week old *Hfe*^{LysMCre} mice were collected, homogenized and passed through the cell strainer (70μ, BD Biosciences, Germany) and centrifuged for 5minutes at 1500rpm, 4^{0}C. Whole blood was first passed through the erylysis buffer and the cell suspension collected with 3 volumes of FACS buffer (PBS/2%FCS/2mM EDTA) and centrifuged at 1500rpm, 4^{0}C for 5minutes. Cells were washed, resuspended in FACS buffer and counted under microscope using hemocytometer. After 10 minutes of incubation in Fc-block (1:200; BD, USA), cells were stained with following antibodies and respective isotypes: CD3-FITC (clone: 142-2c11), CD4-PB (clone: RM4-5), CD8-Apc-Cy7 (clone: 53-6.7), CD19-PE-Cy7 (clone: eBio1D3), CD11b-FITC (clone: M1/70), F4/80-PE (clone: BM8), CD45-APC (clone: 30-F11), B220-perCP Cy5.5 (clone: RA3-6B2) and MHCII-AF700 (clone: M5/114.15.2), all from eBioscience, Germany; Ly6G-PE-Cy7 (clone: RB6-8C5, Invitrogen, USA). A minimum of 10,000 events was counted with an LSRII flow cytometer (BD Biosciences, CA, USA) and analysis performed by FlowJo software (Treestar, OR, USA).

Generation of vector containing Hfe cDNA, plasmid isolation and transfection into BMDM

A full-length 1.1 kb mouse *Hfe* cDNA (sequence information retrieved from NCBI reference sequence: NM_010424.5) fragment tagged with myc epitope was amplified using the *Hfe* -myc forward (5'-GCAATGGCTACAGGGTGAC-3') and reverse primers (5'-CTCTCTTCCGTGGTTCAGC-3'). The fragment was then subcloned into pcDNA6.2 vector backbone using the *Nhe*1 and *BamH*1 restriction sites. The cloned product of *Hfe*-myc was further transformed into DH5-alpha cells via electroporation (1.8kV, 9.10 milli second, 1mm gap). Correct orientation and sequence were confirmed by restriction cleavage and sequencing analysis. BMDMs were transfected with vector carrying *Hfe*-myc or with empty vector for 24 hours using Attractene reagent (Qiagen, USA). The delivery of *Hfe* into BMDMs was confirmed

by real time qPCR analysis for the *Hfe*.

RNA isolation, Reverse-transcription and real-time PCR

Total RNA was isolated from tissues or cells using Trizol reagent (Invitrogen, Ambion, USA) or RNeasy Midi kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Data were calculated using delta delta Ct (ΔΔCt) method and the relative mRNA expression of the gene of interest was normalized to the reference gene *Gapdh*. The primers used are:

Gene	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
Slc40a1(Fpn1)	TGTCAGCCTGCTGTTTGCAGGA	TCTTGCAGCAACTGTGTCACCG
Tfr1	CCCATGACGTTGAATTGAACCT	GTAGTCTCCACGAGCGGAATA
Hamp1	ATACCAATGCAGAAGAGAAGG	AACAGATACCACACTGGGAA
Id1	ACCCTGAACGGCGAGATCA	TCGTCGGCTGGAACACATG
Tfr2	GGAGGTCAATTCCCATACCCT	CGACCACCAACACGGAGTC
Dmt1	AGCTAGGGCATGTGGCACTCT	ATGTTGCCACCGCTGGTATC
Bmp6	GTGACACCGCCACAAC	TCGTAAGGGCTCTCTG
Smad7	GCAGGCTGTCCAGATGCTGT	GATCCCCAGGCTCCAGAAGA
Hmox1	AGGCTAAGACCGCCTTCCT	TGTGTTCCTCTGTCAGCATCA
Pai1	TGCATCGCCTGCCATTG	CTTGAGATAGGACAGTGCTTTTTCC
Tnfα	TGCCTATGTCTCAGCCTCTTC	GAGGCCATTTGGGAACTTCT
Il1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
iNOS	GAGACAGGGAAGTCTGAAGCAC	CCAGCAGTAGTTGCTCCTCTTC
Cox2	TCTTTGCCCAGCACTTCAC	ACACCTCTCCACCAATGACC
Il10	TCGGAAATGATCCAGTTTTAC	TCACTCTTCACCTGCTCCAC

Ifnγ	TAGCCAAGACTGTGATTGCGG	AGACATCTCCTCCCATCAGCAG
Ym1	CTGGGTCTCGAGGAAGCC	AGTGAGTAGCAGCCTTGGAA
Il1α	ACGGCTGAGTTTCAGTGAGACC	CACTCTGGTAGGTGTAAGGTGC
Tlr1	GGTAGCAAGAGAAGTGGTGGAG	CGATGGTGACAGTCAGCAGAAC
Tlr2	ACAGCAAGGTCTTCCTGGTTCC	GCTCCCTTACAGGCTGAGTTCT
Tlr3	GTCTTCTGCACGAACCTGACAG	TGGAGGTTCTCCAGTTGGACCC
Tlr4	AGCTTCTCCAATTTTTCAGAACTTC	TGAGAGGTGGTGTAAGCCATGC
Tlr5	TCCTGACCAGAGCACATTTGCC	CCTTCAGTGTCCCAAACAGTCG
Tlr6	GTGAGGATGCTGTGTCAGTGGA	CCAGGCAGAATCATGCTCACTG
Tlr7	GTGATGCTGTGTGGTTTGTCTGG	CCTTTGTGTGCTCCTGGACCTA
Tlr8	AAGTGCTGGACCTGAGCCACAA	CCTCTGTGAGGGTGTAAATGCC
Tlr9	GCTGTCAATGGCTCTCAGTTCC	CCTGCAACTGTGGTAGCTCACT
Tlr11/12	GGAGACCTTGACTATAAGCGGC	GCAGAAGGCATCCAAGCTGTTC
Myd88	GAGGATATACTGAAGGAGCTGAAGTC	CCTGGTTCTGCTGCTTACCT
Cd14	TTGAACCTCCGCAACGTGTCGT	CGCAGGAAAAGTTGAGCGAGTG
Gapdh	CCCATTCTCGGCCTTGACTGT	GTGGAGATTGTTGCCATCAACGA

<u>Protein isolation and Western blot analysis</u>

Protein extracts were prepared from flash-frozen tissue or BMDMs after homogenization in RIPA lysis buffer or Cell lysis buffer kit (Bio-Rad Laboratories, USA) supplemented with the protease and phosphatase inhibitors. A total of 30-50μg of proteins was subjected to western blot analysis with the following antibodies: Rabbit-anti-Ferroportin (Alpha Diagnostics Ltd., USA; 1:500), Mouse anti-TfR1 (Zymed laboratories, USA; 1:500), Rabbit anti-pSMAD1/3 (Abcam,

USA; 1:1000), Rabbit anti-Ferritin H (Cell signaling Technology, USA; 1:1000 in 2% BSA), Rabbit anti-total SMAD1 (Cell signaling Technology, USA; 1:1000) and anti-β-actin (Sigma Aldrich, USA; 1:10,000) as loading control. Furthermore, membranes were washed and incubated with anti-rabbit (Invitrogen, USA; 1:5000) or anti-mouse (Invitrogen, USA; 1:5000 or 1:10,000) horseradish peroxidase-conjugated antibody. Western blot images were acquired using EMD Millipore Luminata HRP chemiluminescence substrate (Millipore, USA) and signal acquired in Bio-Rad chemiluminescent detector (Bio-Rad Laboratories, USA). The signals quantified by scanning densitometry and computer-assisted image analysis (ImageJ; www://rsb.info.nih.gov/ij/).

Phosphoprotein analysis using Bio-Plex Pro Cell Signaling MAPK Panel

Protein lysates (10 μg in 50 μl) were prepared from BMDMs using Bio-Plex Cell Lysis Kit (BioRad Laboratories, Germany). The levels of nine intracellular phosphoproteins including MEK1 (Ser217/Ser221), ERK1/2 (Thr202/Tyr204, Thr185/Tyr187), as classical MAPK backbone components, as well as stress-responsive MAPKs, such as JNK (Thr183/Tyr185), (Thr180/Tyr182), and p38 MAPK (Thr180/Tyr182), and effector molecules of MAPKs including p90 RSK (Ser380), STAT3 (Ser727), ATF-2 (Thr71), HSP27 (Ser78) and p53 (Ser15), were measured using Bio-Plex Pro Cell Signaling MAPK Panel, 9-plex (BioRad Laboratories, Germany) according to the manufacturer's instructions. The data were analyzed using Bio-Plex Manager 6.1 Software Package.

Statistical analyses

Data were analyzed using Prism v.5 (GraphPad) and presented as mean ± SEM (standard error of mean). For the statistical analysis a one-way analysis of variance (ANOVA) with Tukey's multiple comparison was used to measure the difference between groups. For pairwise

comparisons the Student *t*-test (two-tailed; unpaired; unequal variance) was used. Kaplan-Meier survival curve was plotted using Prism 5 (GraphPad Software) and differences between groups were determined using log-rank test. *p<0.05, **p<0.01, ***p<0.005, ****p<0.001, are considered statistically significant.

References:

1. Vujić Spasić M, Kiss J, Herrmann T, et al. Hfe acts in hepatocytes to prevent hemochromatosis. Cell Metab. 2008;7(2):173-8.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Lack of *Hfe* in macrophages does not affect hematopoietic cell composition

(**A, B**) Percentage of T-, B-lymphocytes and macrophages in the bone marrow and the spleen of 45-week old $Hfe^{LysMCre}$ mutant mice in regard to controls (*n*=6; 6). Data are shown as mean values ± SEM. *n* indicates the number of mice used in the analysis.

Supplementary Figure 2. Serum hepcidin and cytokine profile in 45-week old $Hfe^{LysMCre}$ mutant mice during LPS-induced endotoxin shock

Hepcidin and the inflammatory cytokine and chemokine levels were examined in the sera of LPS (10 mg/kg) injected 45-week old $Hfe^{LysMCre}$ mutant and control mice (*n*=11;11) with Hepcidin ELISA and Bio-Plex Pro mouse Cytokine panel 23-plex kit. Pattern analysis of hepcidin (**A**) and immune mediators in mutant $Hfe^{LysMCre}$ mice (indicated in red) during the course of LPS injections, whereby some expression levels were (**B**) comparable, (**C**) increased or (**D**) decreased relative to control Hfe^{flox} mice (indicated in black).

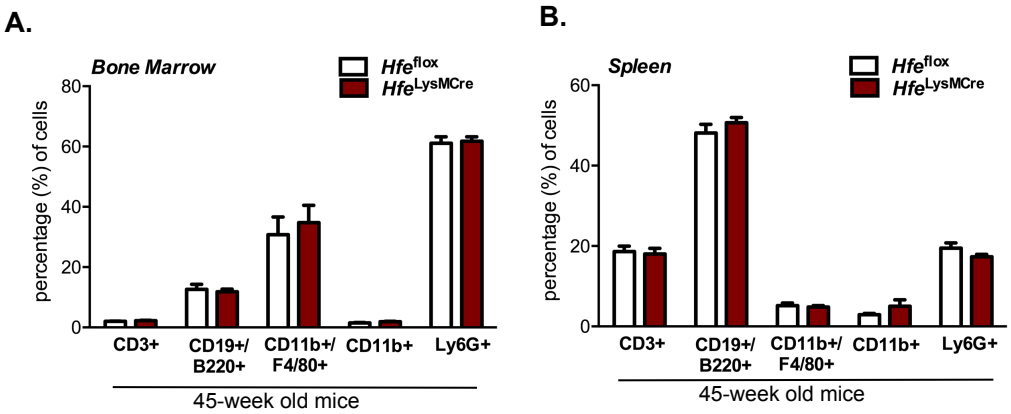
Data are shown as mean ± SEM. Statistically significant differences between LPS-treated and control groups are indicated in red for $Hfe^{LysMCre}$ mice and in black for control mice as **p*<.05, ***p*<.005, ****p*<.0005.

Supplementary Figure 3. Iron responses in the liver and the spleen of mice kept on an iron-deficient diet

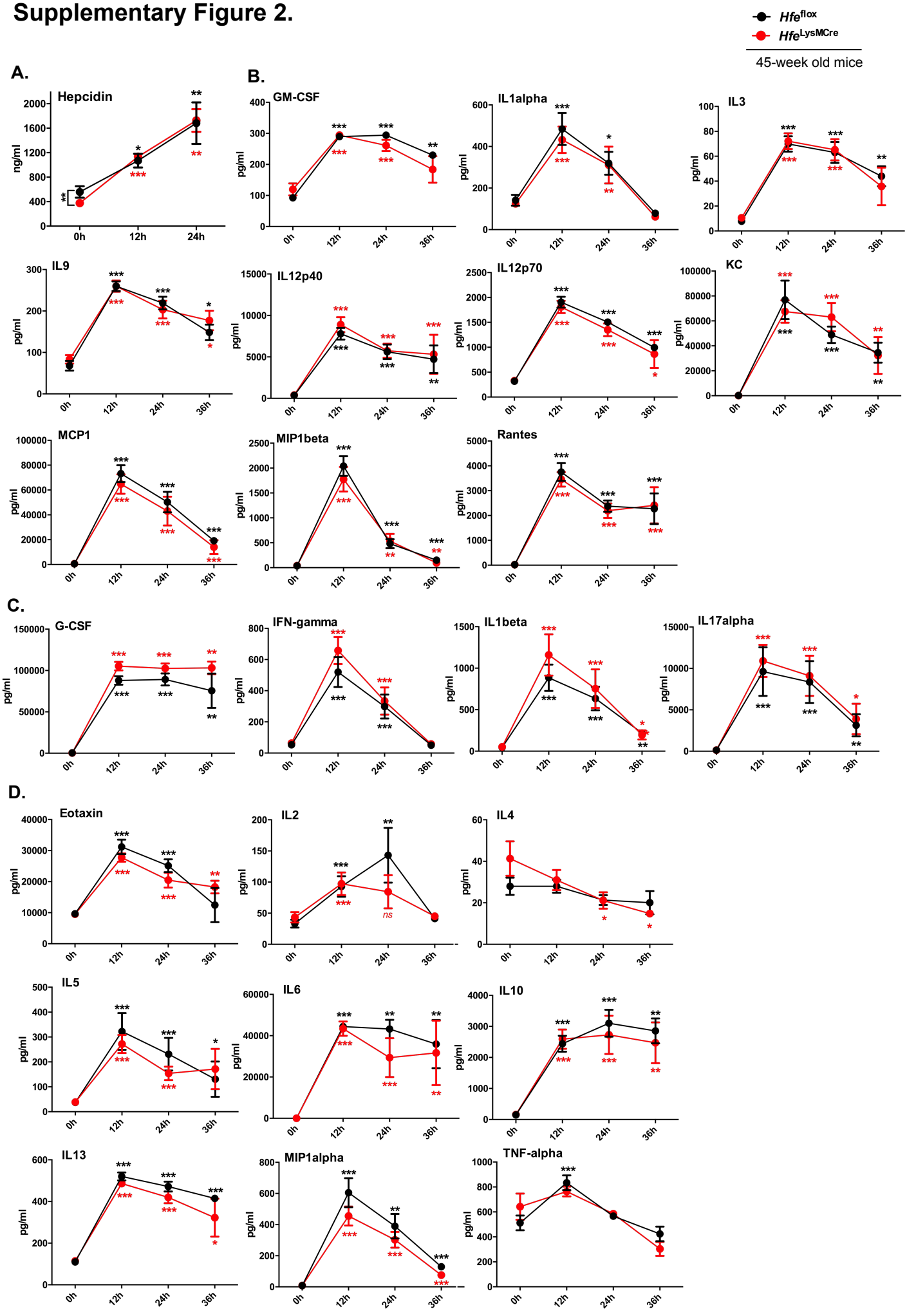
(**A, B**) Representative immunoblot analysis of TfR1 and Ferroportin (FPN), relative to β-actin levels (shown in histograms below), in the livers and spleens of mice maintained on an iron-deficient diet for 4 weeks (IDD). M: Prestained Protein Marker PageRuler (Thermo

Scientific). Data are shown as mean ± SEM. Statistically significant differences are indicated as *p<.05, **p<.005.

Supplementary Figure 1.



Supplementary Figure 2.



Supplementary Figure 3