Cell-specific expression of *Hfe* determines the outcome of *Salmonella enterica* serovar Typhimurium infection in mice

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SUPPLEMENTARY APPENDIX

Supplementary Information

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Supplementary Methods

Salmonella strains

Unless otherwise specified, we used the *S.* Tm. *WT* strain ATCC 14028s for infection experiments. In some experiments, isogenic mutant derivatives deficient for either of the three (*entC::aph*, Δ*sit::bla* and Δ*feo::Tn10* (Tet^r), respectively) or all three (*entC::aph* Δ*sit::bla* Δ*feo::Tn10* (Tet^r)) iron acquisition pathways were additionally used. The construction of these strains has been described (27).

Salmonella infection *in vivo*

Mice were back-crossed on a C57BL/6J genetic background for at least 10 generations and housed in the EMBL animal facility under a constant light-dark cycle with *ad libitum* access to food and water. All mouse breeding were approved by and conducted in compliance with the guidelines of the EMBL Institutional Animal Care and Use Committee. Following infection, mice were monitored twice daily for signs of illness and moribund mice were sacrificed. The bacterial load of organs was determined by plating serial dilutions of organ homogenates on LB agar (Sigma-Aldrich) under sterile conditions and the number of bacteria was calculated per gram of tissue.

Measurement of and protein concentrations

Cytokine (IL-6 and IFN-γ) and Lcn2 levels in sera and tissues were determined by specific ELISA sets (obtained from BD Biosciences for cytokines and R&D, respectively, for Lcn2). Serum levels of hepcidin-1 were measured by a specific ELISA set (Intrinsic Lifesciences), too. Also, levels of other proteins in tissues were quantified by specific ELISA sets (Fpn1 and Tfr1 kits were from Wuhan Fine biotech, the FTH kit was from Blue Gene, the Nos2 kit was purchased from Antikoerper Online) according to the manufacturers' recommendations. To this end, tissue extracts were prepared in cytoplasmatic lysis buffer (25 mM Tris-HCl, pH 7.4, 40 mM KCl, 1% Triton X-100) containing 1 mg/ml aprotinin and 1 mg/ml leupeptin (all obtained from Sigma-Aldrich). Extracts were diluted 50 to 50,000 fold in PBS before use in the respective ELISA. To ensure accurate comparisons of tissue protein levels, ELISA results were normalized for the total protein content as assessed by the Bradford method.

Histopathology

Histologic examination of spleens and livers was performed on formalin-fixed tissue sections automatically stained with H&E or for Prussian blue according to standard protocols. For image acquisition, a Zeiss Axioskop 2 microscope equipped with a 5x objective with a numerical aperture of 0.12, an AxioCam MRc5 digital camera and the AxioVision Rel. 4.8 software were used.

In vitro experiments

For the isolation of bone marrow-derived macrophages (BMDM), age- and sex-matched mice were sacrificed. Bone marrow cells were recovered from tibiae and femora and erythrocytes were lysed by two rounds of incubation for 10 min in RBC Lysis Buffer (Biolegend) and extensive washing in PBS as described (26). BMDM were enriched by plastic adherence and differentiated using 100 ng/ml recombinant murine (rmu) M-CSF (Prepotech). After 6 to 8 days in culture, BMDM were transferred to 6-well plates and grown overnight. Thereafter, BMDM were left untreated, stimulated with LPS and/or exposed to fluorescence-labelled bacteria (pHrodo® Green BioParticles; Life Technologies, ThermoFisher Scientific) and incubated at 4°C (unspecific surface binding) or 37°C (phagocytosis) for 15 min. After extensive washing in PBS, phagocytosis was assessed by flow cytometry.

Sequences of primers and probes

Primers and probes, carrying 5'FAM and 3'BHQ1 labels, were obtained from Microsynth. Sequences were as follows (primer forward, primer reverse, probe):
Dmt1: 5'-GGACTGTGGACGCTCGGTAA-3', 5'-AATGTTGCCACCGCTGGT-3', 5'-CATCTCGAAAGTCCTGCTGAGCGAAGA-3', *Fpn1*: 5'-CTACCATTAGAAGGATTGACCAGCT-3', 5'-CAAATGTCATAATCTGGCCGA-3', 5'-CAACATCCTGGCCCCCATGGC-3', *Il-6*: 5'-TGTTCTCTGGGAAATCGTGGA-3', 5'-AAGTGCATCATCGTTGTTCATACA-3', 5'-ATGAGAAAAGAGTTGTGCAATGGCAATTCTG-3', *Il-10*: 5'-CCAGAGCCACATGCTCCTAGA-3', 5'-TGGTCCTTTGTTTGAAAGAAAGTCT-3', 5'-TGCGGACTGCCTTCAGCCAGG-3', *Ifn-γ*: 5'-TCAAGTGGCATAGATGTGGAAGAA-3', 5'-TGGCTCTGCAGGATTTTCATG-3', 5'-TCACCATCCTTTTGCCAGTTCCTCCAG-3', *Lcn2*: 5'-GCCTCAAGGACGACAACATCA-3', 5'-TTCTCTGTCCCCACCGACCAATGC-

3’, 5’-CACCACCCATTCAGTTGTCAAT-3’, *Hamp1*: 5’-GGCAGACATTGCGATACCAAT-3’, 5’-TGCAACAGATACCACACTGGGAA-3’, 5’-CCAACTTCCCCATCTGCATCTTCTGC-3’, *H-Ft*: 5’-GCGAGGTGGCCGAATCT-3, 5’-CAGCCCGCTCTCCCAGT-3’, 5’-CCTGCAGGATATAAAGAAACCAGACCGTGA-3’, *Hmox1*: 5’-GTGATGGAGCGTCCACAGC-3’, 5’-TGGTGGCCTCCTTCAAGG-3’, 5’-CGACAGCATGCCCCAGGATTTGTC-3’, *Hprt*: 5’-GACCGGTCCCGTCATGC-3’, 5’-TCATAACCTGGTTCATCATCGC-3’, 5’-ACCCGCAGTCCCAGCGTCGTC-3’, *Nos2*: 5’-CAGCTGGGCTGTACAAACCTT-3’, 5’-CATTGGAAGTGAAGCGTTTCG-3’, 5’-CGG GCA GCC TGT GAG ACC TTT GA-3’, *Phox-p47*: 5’-CAAAGATGGCAAGAATAACGTAGC-3’, 5’-AGTCAGCAATGGCCCGATAG-3’, 5’-CATCACAGGCCCCATCATCCTTCAGA-3’, *Tfr1*: 5’-CGCTTTGGGTGCTGGTG-3’, 5’-GGGCAAGTTTCAACAGAAGACC-3’, 5’-CCCACACTGGACTTCGCCGCA-3’, *Tnf*: 5’-TTCTATGGCCCAGACCCTCA-3’, 5’-TTGCTACGACGTGGGCTACA-3’, 5’-CTCAGATCATCTTCTCAAAATTCGAGTGACAAGC-3’.

Legends to Supplementary Figures

Supplementary Figure S1. *Alb-Cre*$^{+}$ mice not carrying *loxP* (*Hfe*$^{WT/WT}$) sites have normal control of *Salmonella* infection.

Survival (A) and bacterial load in spleen (B) , liver (C) and serum (D) of mice with insertion of the *Cre*-gene into the hepatocyte-specific albumin (*Alfp*) promoter region (*AlfpCre*$^{+}$ *Hfe*$^{WT/WT}$) and their respective controls (*AlfpCre*$^{-}$ *Hfe*$^{WT/WT}$). Mice were infected with 500 CFU of *S. enterica* serovar Typhimurim by intraperitoneal injection. Data represent two independent experiments. *n* = 16 for *AlfpCre*$^{-}$ *Hfe*$^{WT/WT}$, 20 for *AlfpCre*$^{+}$ *Hfe*$^{WT/WT}$. Statistics: survival data (A) between transgenic and control mice were compared using the Log-rank (Mantel-Cox) Test. Log CFU data of tissue bacterial load (B and C) of transgenic and control mice were compared using student *t*-test. We used Mann-Whitney testing to compare bacterial numbers in serum. No statistically significant differences were observed. *n* = 10 for *AlfpCre*$^{-}$ *Hfe*$^{WT/WT}$, 10 for *AlfpCre*$^{+}$ *Hfe*$^{WT/WT}$.

Supplementary Figure S2. Absence of *Hfe* in hepatocytes causes parenchymal iron accumulation in the liver.

Liver sections of *Hfe*$^{-/-}$ mice (A), *AlfpCre*$^{+}$ mice (C) and *LysMCre*$^{+}$ mice (E) infected for 72 hrs were stainined by Prussian blue to assess iron distribution. Scale bars: 200 µM.

Hepatic iron content (B, D and F) was measured and normalized for protein content. Data were compared by Mann-Whitney test. *n* = 12 for *Hfe*$^{+/+}$, 12 for *Hfe*$^{-/-}$, 20 for *AlfpCre*$^{-}$ *Hfe*$^{fl/fl}$, 14 for *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$, 9 for *LysMCre*$^{-}$ *Hfe*$^{fl/fl}$, 9 for *LysMCre*$^{+}$ *Hfe*$^{fl/fl}$.

Supplementary Figure S3. Hfe does not affect phagocytosis.

The phagocytic capacity of *Hfe*$^{+/+}$ and *Hfe*$^{-/-}$ BMDM was assessed following incubation with FITC-labeled bacteria and addition of PBS or LPS at 37°C for 15 min by flow cytometry. Cells not exposed to FITC-labeled bacteria served as blank. The FITC-signal intensity of cells kept on ice (4°C) marks unspecific binding to the cell surface and uptake. One of three representative experiments is shown in histograms depicting fluorescence intensity.

Supplementary Figure S4. Dietary iron overload does increase the bacterial load in spleen and liver.

Serum samples of *AlfpCre*$^{+}$ mice infected with *S.* Tm. for 72 hrs were used to measure serum GPT activity (A). Data were compared by Mann-Whitney testing.

Bacterial load in spleen (B) and liver (C) of C57BL/6 mice fed an iron-adequate (IA) diet or subjected to oral iron overload (IO) with an iron-enriched diet containing 25 mg/g of iron 3 weeks prior and during infection. Mice were infected with 500 CFU of *S.* Tm. by intraperitoneal injection. Log CFU data of two independent experiments were compared using student *t*-test. $n = 8$ for IA, $n = 8$ for IO.

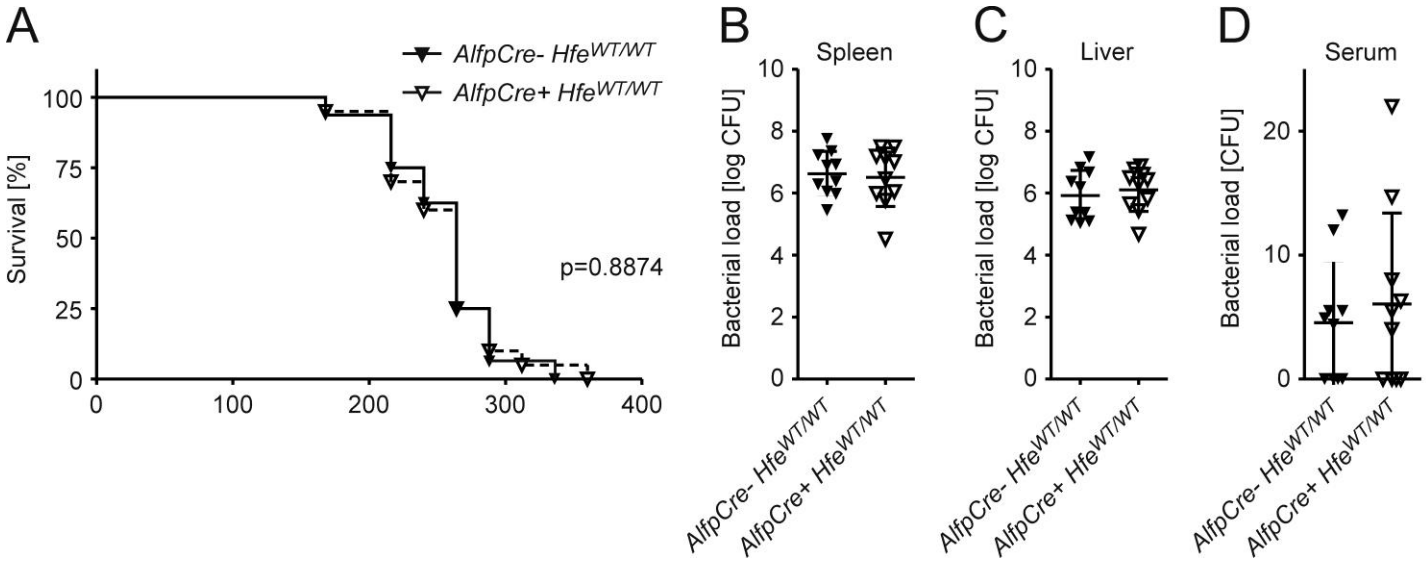
Supplementary Figure S5. Hepatic protein expression as a function of cell-type specific *Hfe*-deletion.

Protein levels of Fpn1 (A), H-Ft (B), Tfr1 (C), Lcn2 (D) and IL-6 (E) were measured by ELISA and normalized for protein content. Data are presented and were compared as in fig. 4. $n = 10$ for *Hfe*$^{+/+}$, 12 for *Hfe*$^{-/-}$, 20 for *AlfpCre*$^{-}$ *Hfe*$^{fl/fl}$, 14 for *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$, 15 for *LysMCre*$^{-}$ *Hfe*$^{fl/fl}$, 15 for *LysMCre*$^{+}$ *Hfe*$^{fl/fl}$.

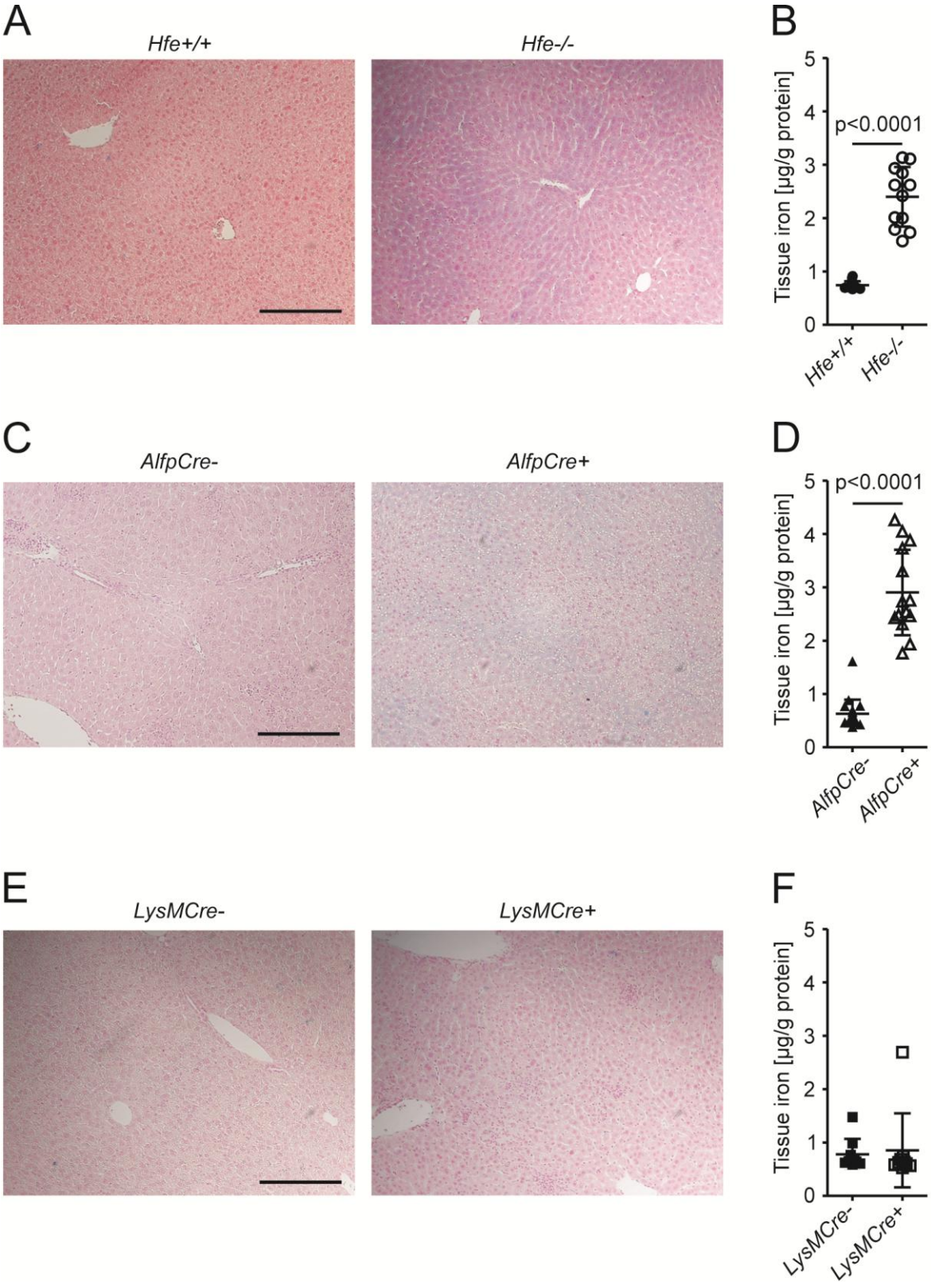
Supplementary Figure S6. Cell-type specific *Hfe*-deficiency determines outcome of *Salmonella* infection.

Compared to *WT* mice (n denotes normal) expressing *Hfe* in all cell types (+), *Salmonella*-infected mice with *Hfe*-deficiency (-) in macrophages (global or specific) show reduced bacterial numbers (↓) in spleen and liver, increased *Nos2* expression and increased survival time. *Salmonella*-infected mice with hepatocyte-specific *Hfe*-deficiency or mice with dietary iron overload show elevated serum iron levels and higher bacterial burden (↑) in the serum as well as reduced IFN-γ production in the spleen and liver.

Supplementary Figure S1.



Supplementary Figure S2.



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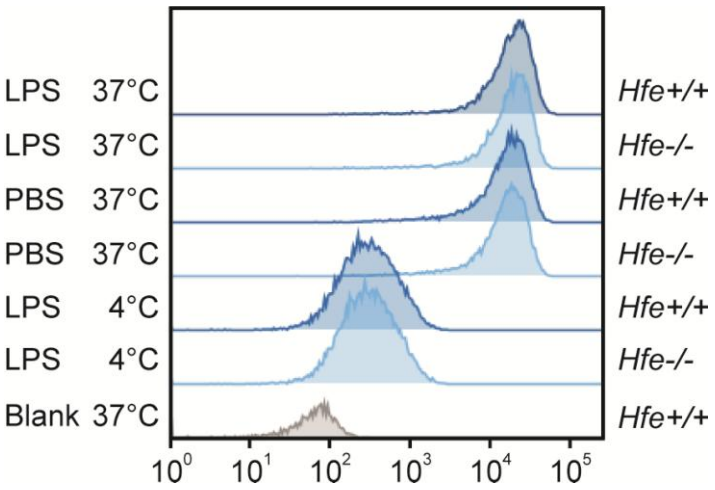

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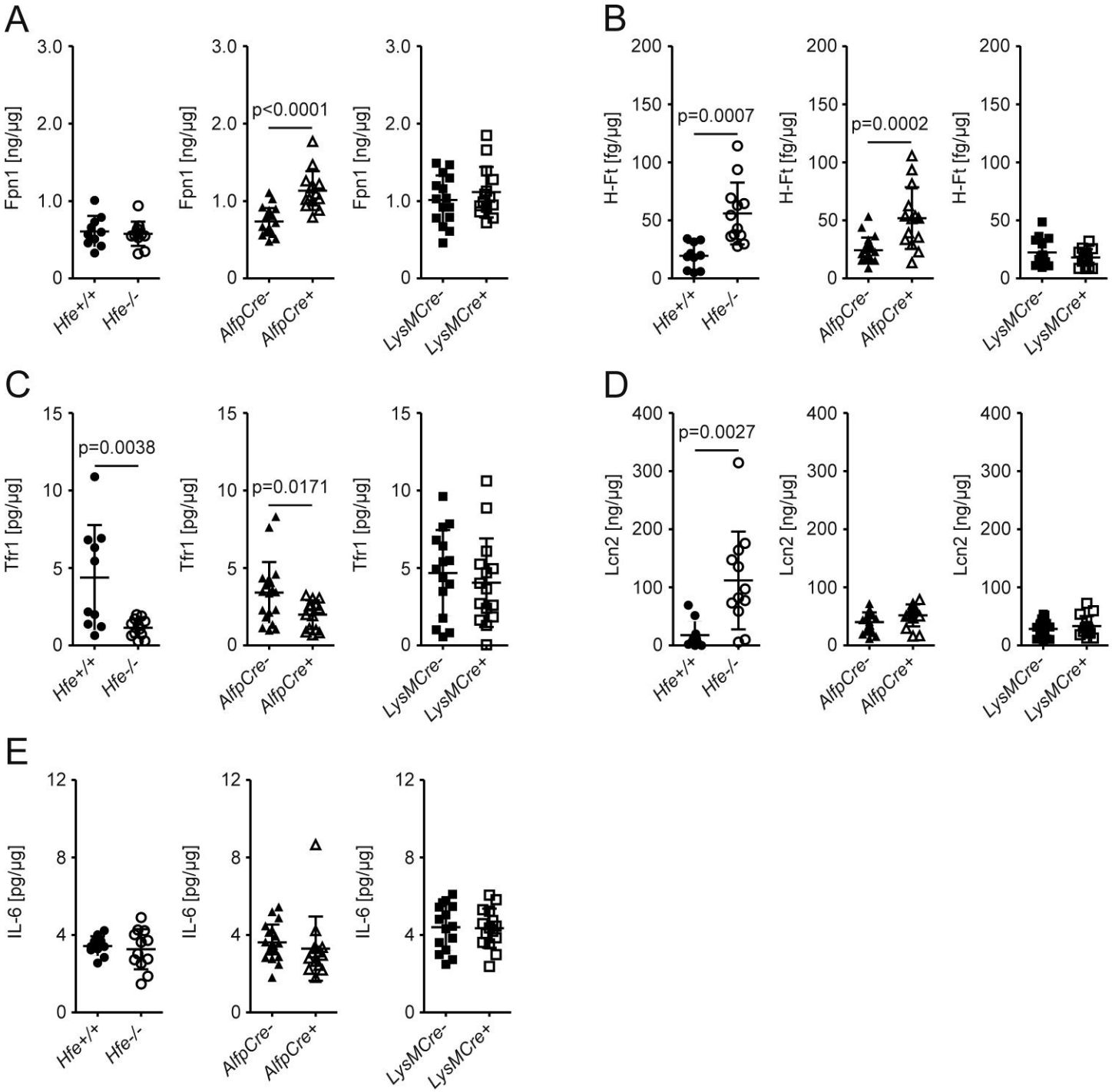
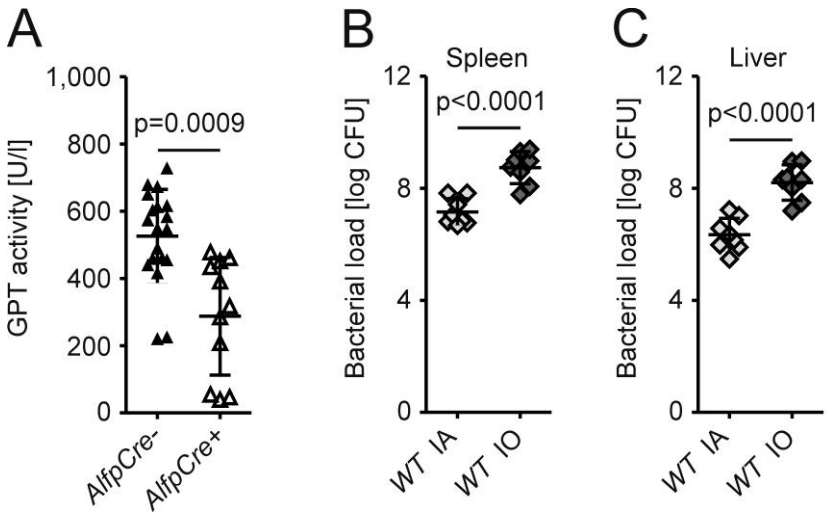


Supplementary Figure S5.



Supplementary Figure S6.