Normal systemic iron homeostasis in mice with macrophage-specific deletion of transferrin receptor 2

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Running head: Macrophage Tfr2 is not required for systemic iron homeostasis

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Abstract

Iron is an essential element, as it is a component of many macromolecules involved in diverse physiological and cellular functions, including oxygen transport, cellular growth and metabolism. Systemic iron homeostasis is predominantly regulated by the liver through the iron regulatory hormone hepcidin. Hepcidin expression is itself regulated by a number of proteins including transferrin receptor 2 (TFR2). TFR2 has been shown to be expressed in the liver, bone marrow, macrophages and peripheral blood mononuclear cells. Studies from our laboratory have shown that mice with a hepatocyte-specific deletion of \textit{Tfr2} recapitulate the hemochromatosis phenotype of the global \textit{Tfr2} knockout mice, suggesting that the hepatic expression of TFR2 is important in systemic iron homeostasis. It is unclear how TFR2 in macrophages contributes to the regulation of iron metabolism. We examined the role of TFR2 in macrophages by analysis of transgenic mice lacking \textit{Tfr2} in macrophages by crossing \textit{Tfr2}^{f/f} mice with \textit{LysM-Cre} mice. Mice were fed an iron-rich diet or injected with lipopolysaccharide to examine the role of macrophage \textit{Tfr2} in iron- or inflammation-mediated regulation of hepcidin. Body iron homeostasis was unaffected in the knockout mice, suggesting that macrophage TFR2 is not required for the regulation of systemic iron metabolism. However, peritoneal macrophages of knockout mice had significantly lower levels of ferroportin mRNA and protein, suggesting that TFR2 may be involved in regulating ferroportin levels in macrophages. These studies further elucidate the role of TFR2 in the regulation of iron homeostasis and its role in regulation of ferroportin and thus macrophage iron homeostasis.

**Keywords:** Transferrin receptor 2, iron metabolism, hepcidin, macrophages.
Introduction

Iron is an essential mineral; it is required for the functioning of a variety of proteins and enzymes. A deficiency of iron leads to anemia, which has been recognized by the World Health Organisation as the most common nutritional disorder affecting approximately 2 billion people world-wide (5, 20). Excess iron leads to hemochromatosis, which is characterized by iron deposition in the tissues and ultimately leads to organ dysfunction (4). In the absence of a known excretory mechanism it is essential for the body to regulate the amount of iron absorbed. Central to this regulation is hepcidin, a 25 amino acid peptide produced predominantly in the liver, encoded by the HAMP gene. Hepcidin binds to and induces the internalization of ferroportin (FPN) the only known iron export protein, thus limiting the amount of iron released into the bloodstream.

Hepcidin is regulated in response to a number of external and internal stimuli, including but not limited to body iron stores, inflammation, erythropoiesis and hypoxia (30). These stimuli have been shown to mediate hepcidin synthesis through several different molecules including the hemochromatosis protein (HFE), hemojuvelin (HJV), transferrin receptor 2 (TFR2), interleukin 6 (IL6), and erythropoietin (reviewed in (30)). Patients (6, 33) and mice (9, 40) with mutations in TFR2 develop type 3 hemochromatosis which is characterized by inappropriate hepcidin levels in relation to body iron stores (24, 38). The mechanism by which TFR2 mediates hepcidin regulation is still unclear. Previous studies have reported that TFR2 can interact with HFE (10, 11), and that this interaction is required for the regulation of hepcidin (10). Recently it was proposed that TFR2, HFE and the bone morphogenetic protein (BMP) co-receptor HJV can form a multi-protein complex which regulates HAMP levels in response to increased iron levels in human
hepatoma HepG2 cells (8). Contrary to this, studies using the Hfe/Tfr2 double knockout mice (37) or transgenic mice expressing myc-tagged HFE (34) have suggested that HFE and TFR2 can act independently to regulate hepcidin. Recent studies from our laboratory have also suggested that HFE and TFR2 do not interact in a stable co-expression system (29). Although the mechanism is still unclear, as a result of these studies we know that TFR2 in hepatocytes is required for an appropriate HAMP response.

TFR2 is predominantly expressed in the liver (16), but initial studies had also reported mRNA expression in extra-hepatic tissues including bone marrow, spleen, peripheral blood mononuclear cells, prostate gland (14, 16) and splenic and peritoneal macrophages (32). Mice with a hepatocyte-specific deletion of Tfr2 recapitulate the iron overload phenotype of the global Tfr2 knockout mice (9, 38-40), suggesting that the hepatic expression of TFR2 is sufficient for the systemic regulation of iron homeostasis. However, it is unclear how and whether TFR2 expressed in macrophages contributes to the regulation of iron metabolism. In order to examine the role of TFR2 in macrophages we generated transgenic mice lacking Tfr2 in macrophages by crossing Tfr2<sup>f/f</sup> mice with LysM-Cre transgenic mice. The role of macrophage TFR2 in systemic iron metabolism, in the presence of excess iron or an inflammatory stimulus, was also determined by feeding the mice an iron-rich diet or injecting them with lipopolysaccharide (LPS). While the results from this study indicate that macrophage TFR2 is not required for systemic iron homeostasis, they also suggest that Tfr2 expression in the macrophages may be required for the regulation of Fpn1, an essential component of the iron transport pathway.
Materials and Methods

Generation of Tfr2f/f/LysM-Cre+/- mice and treatments

All animal experimentation was performed according to the guidelines and approval of the QIMR Berghofer Medical Research Institute Animal Ethics Committee. Mice were housed under a 12 hour light/dark cycle and were provided with food and water ad libitum. Tfr2f/f mice (40) were bred with Tfr2+/+/LysM-Cre+/- mice to generate control (Tfr2f/f/LysM-Cre-/+) and macrophage-specific knockout (KO) (Tfr2f/f/LysM-Cre+/+) mice. Control and KO male mice at 3 weeks of age were fed a control (iron content: 68 mg/Kg) or an iron-rich (iron content: 20 g/Kg) diet for 2 weeks (Specialty Feeds, Glen Forest, Western Australia). 5-week-old control and KO male mice were injected with LPS (1 μg/g of body weight) (Sigma-Aldrich, Sydney, NSW, Australia) or saline (control) for 6 hours and their tissues harvested for further analysis. All mice used in this study were bred on a C57BL/6J background.

Isolation and culture of peritoneal macrophages

Peritoneal macrophages were isolated using the peritoneal gavage method as described (43). Anaesthetized control and KO mice (n=5 and n=3 respectively) were injected intraperitoneally with 10 ml of ice cold phosphate buffered saline (PBS). Using the same syringe and needle the fluid was aspirated from the peritoneum. The cells were then centrifuged at 4°C for 10 min at 400Xg. After centrifugation, the cell pellet was resuspended in RPMI 1640 medium (Life Technologies, Mulgrave, Australia) supplemented with 10% fetal calf serum (FCS) (Hyclone Lab. Inc., Mordialloc, Victoria, Australia). Freshly isolated cells were seeded at a concentration of 300,000 cells per well in a 12 well tissue culture plate in RPMI medium supplemented with 10% FCS. The cells were allowed to adhere to the plastic surface.
for 3–4 hr, and non-adherent cells were removed by exchanging the medium. After overnight incubation at 37°C in a 5% CO2 atmosphere, cells were collected and used for RNA or protein isolation.

Tissue and serum iron indices
Serum iron indices were measured using the iron/total-iron binding capacity reagent (Pointe Scientific, Canton, MI, USA). Splenic (SIC) and hepatic (HIC) iron concentrations were measured using the method of Torrance and Bothwell (35).

Histology
Formalin (10%) fixed tissues were processed, paraffin embedded and sectioned by the QIMR Berghofer Histotechnology Facility. Perls’ staining was performed as described by McDonald et al (19). Slides were scanned using the Aperio AT Turbo (Aperio, Vista, CA, USA) at 20X magnification. The sections were analyzed using ScanScope software (Aperio).

Real time PCR
Total RNA was isolated from bone marrow, kidney, liver, spleen or peritoneal macrophages using TRIzol reagent (Life Technologies). SensiFAST™ cDNA synthesis kit (Bioline Pty Ltd, Sydney, NSW, Australia) was used to prepare cDNA from 1 µg of total RNA. Real time quantitative-PCR (qPCR) was performed using the SensiFAST™ SYBR No-Rox kit (Bioline) and the following conditions: 5 minutes denature at 95°C followed by 45 cycles of 95°C for 15 seconds, 60°C for 10 seconds and 72°C degrees for 15 seconds. The expression of all target genes was normalized to the geometric mean of three reference genes: β-actin (Actb),
hypoxanthine-guanine phosphoribosyl transferase (Hprt) and DNA-directed RNA polymerase II subunit RPB1 (Polr2a). Primers sequences are listed in Table 1.

**Western Blotting**

Liver tissue (50-100 mg) was homogenized in protein extraction buffer (described previously (29)) using the Precellys™ 24 tissue homogenizer. Peritoneal macrophages were harvested directly in 200 µl of the protein extraction buffer. Tissue homogenates (25 µg protein) or cell lysates (50 µl) were electrophoresed on 12% sodium dodecyl sulfate polyacrylamide gels at 200 V for 1 hour 15 mins. The proteins were then transferred onto nitrocellulose membranes (0.2 µm pore size) (Bio Rad Laboratories P/L, Gladesville, NSW, Australia) using the Trans-blot Turbo blotting apparatus (Bio Rad) at 25 V, 2.5 A for 30 minutes in the transfer buffer. The membrane was then blocked for 2 hours at room temperature (RT) with 10% non-fat milk and then incubated with primary antibodies (anti-actin (1:40,000) (Sigma-Aldrich), anti-TFR2 (1:20,000) (38), anti-pSMAD1/5 (1:2000) (Cell Signaling Technology, Danvers, MA or anti-FPN (1:1000) (Alpha Diagnostics, San Antonio, TX) diluted in 10 % non-fat milk) overnight at 4°C. The following day, membranes were washed and incubated with secondary antibodies (anti-mouse/rabbit heavy and light chain IgG conjugated to horseradish peroxidase (HRP) (1:10,000) (Invitrogen, Life Technologies) diluted in 10% non-fat milk) for 1 hour at RT. After washing the blots were incubated with chemiluminescent substrate (Lumina Forte, Merck Millipore, Kilsyth, Victoria, Australia) for 5 minutes and exposed to X-ray film (Fujifilm, Brookvale, NSW, Australia) for various times. Films were developed using the Minolta film processor (Konica Minolta Medical and Graphic Ltd, Tokyo Japan). The processed films were then scanned using Scanmaker 9800 XL plus (Microtek
International Inc. Hsinchu, Taiwan). Densitometric analysis for quantitation of the proteins was performed using Genetools software (version 4.0) (Syngene, Cambridge, UK).

**Statistical Analyses**

Statistical analyses on variables between different groups of mice was performed by using two-way analysis of variance (ANOVA) and Student's t-test. Post-hoc analysis was performed to compare the differences between individual groups using Tukey's multiple comparison tests. P-values <0.05 were considered to be statistically significant. Statistical analysis was performed using the GRAPHPAD PRISM 6 software (GraphPad Software, San Diego, USA).
Results

Deletion of macrophage Tfr2 leads to a reduction in ferroportin expression in peritoneal macrophages

In order to generate mice lacking Tfr2 specifically in macrophages we crossed mice carrying the floxed Tfr2 allele (39) with mice expressing the Cre recombinase under the lysozyme M promoter that has been shown to be active in macrophages (7). A significant reduction in Tfr2 mRNA in the peritoneal macrophages (p=0.015) of these mice (Tfr2<sup>fl/fl</sup>LysM-Cre<sup>+</sup>- referred to KO mice here) compared to controls was shown by qPCR analysis (Figure 1A), suggesting that the Tfr2 gene had been deleted in these cells. It was previously reported that the β-form of TFR2 may be involved in the regulation of Fpn1 specifically in macrophages, thus specifically controlling iron efflux in the spleen (32). The KO mice used in this study lack both the α- and the β-form of Tfr2, as the loxP sites were inserted flanking exon 2 to exon 6 of the Tfr2 gene (40). The Fpn1 mRNA levels in isolated peritoneal macrophages were significantly lower in the KO mice (Figure 1B).

We also measured the mRNA expression levels of several genes known to be involved directly or indirectly in maintaining macrophage iron homeostasis. There were no significant differences between the mRNA expression levels of divalent metal transporter 1 (Dmt1) (Figure 1C), hypoxia inducible factor 1 α (Hif 1α) (Figure 1D), ferritin heavy chain (Fth) (Figure 1E), ferritin light chain (Ftl) (Figure 1F), superoxide dismutase 2 (Sod2) (Figure 1H) or transferrin receptor 1 (Tfr1) (Figure 1G) in the peritoneal macrophages derived from control and KO mice.

The reduction in Fpn1 mRNA levels was also confirmed at the protein level in the peritoneal macrophages. The amount of FPN protein was significantly reduced in the peritoneal macrophages of the KO mice as compared to the control mice (Figure 2).
Deletion of macrophage Tfr2 does not affect Tfr2 expression levels in the liver or systemic iron parameters

Deletion of Fpn1 in the macrophages of mice has been shown to lead to a mild tissue iron overload in the liver and spleen due to accumulation of iron in macrophages. In order to determine whether the reduction of Fpn1 in the macrophages of the mice lacking macrophage-Tfr2 results in iron overload we examined the liver and systemic iron parameters in the control and KO mice.

The role of macrophage TFR2 in the iron-mediated regulation of systemic iron homeostasis was investigated by measuring serum and tissue iron parameters, and feeding the mice an iron-rich diet (High Fe). There were no significant differences between the serum iron (Figure 3A), transferrin saturation (Figure 3B), hepatic (HIC) (Figure 3C) or splenic iron concentrations (SIC) (Figure 3D) of the control and KO mice. As expected, serum and tissue iron indices increased significantly in the mice fed an iron-rich diet. However, there were no significant differences between the control and KO mice (Figure 3), suggesting that macrophage-specific deletion of Tfr2 does not affect systemic iron levels.

The pattern of iron distribution was assessed by Perls’ staining (Figure 4). No differences in the morphology or the pattern of iron distribution between the control and KO mice was observed, even in mice fed an iron-rich diet. The iron distribution was in the normal periportal pattern, accumulating in hepatocytes of the mice fed an iron-rich diet, with no observable differences between the control and KO mice (Figure 4). In the liver, no stainable iron was observed in either the control or KO mice fed a control diet. Similarly, the spleens of the mice fed an iron-rich diet showed iron loading in the reticuloendothelial cells of the red pulp (Figure 4).
BMP-SMAD mediated-regulation of Hamp in the liver is not affected in KO mice fed an iron-rich diet

It is now clear that Hamp is central to the regulation of iron homeostasis. Hamp itself is regulated in response to a number of external and internal stimuli including body iron levels. The iron-mediated regulation of Hamp is dependent on the BMP-mothers against decapentaplegic homologue (SMAD) pathway (1-3, 21). An increase in body iron levels induces Bmp6 in the liver (13). The binding of BMPs to their receptors induces the phosphorylation of the receptors, which results in a signaling cascade mediating the regulation of downstream genes, including Hamp.

We investigated the role of macrophage TFR2 in the iron-mediated regulation of Hamp by examining the mRNA expression of molecules involved in this pathway in the livers of the control and KO mice fed a control or an iron-rich diet. The mRNA expression levels of Bmp6 (Figure 5A), Hamp (Figure 5B) and the downstream targets of BMP-SMAD signaling, Id1 (Figure 5C) and Smad7 (Figure 5D), were increased in the control and KO mice fed an iron-rich diet. There were no significant differences in the levels of Bmp6 and Hamp and the other downstream targets of the BMP-SMAD signaling pathway between the control and KO groups in their respective diet group (Figure 5A-D), suggesting that iron-mediated Hamp regulation is not affected in the mice lacking TFR2 in macrophages. In addition, we also looked at the mRNA expression of other molecules known to be involved in the iron-mediated regulation of Hamp. There were no significant changes in the mRNA levels of Hfe (Figure 5E) and Hjv (Figure 5F) in the control and KO mice. The levels of Fpn1 in KO mice fed an iron-rich diet were significantly lower compared to the control mice on the same diet (Figure 5G). There were no significant differences in the mRNA levels of Tfr2 in the livers of control and KO mice, suggesting that the
deletion of Tfr2 is specific to the macrophages and has not affected hepatocyte Tfr2 expression (Figure 5H).

In order to analyse the regulation of BMP-SMAD signaling at the protein level, western blotting was performed on total liver homogenates of KO and control mice fed a control or iron-rich diet. There was an increase in the levels of TFR2 protein in the mice fed an iron-rich diet (Figure 6A and B). This was expected, as it has been shown that an increase in holo-TF stabilises the TFR2 protein in cells (12) and liver (31). The levels of pSMAD1/5 were increased in the livers of mice fed an iron-rich diet (Figure 6A and C). These results suggest there is appropriate BMP-SMAD signaling in the livers of the KO mice.

**Deletion of macrophage Tfr2 does not affect inflammation-mediated regulation of Hamp**

The major function of macrophages is to maintain immune homeostasis and one aspect of this involves regulating Hamp in response to inflammation. Inflammatory stimuli such as turpentine and LPS are known to induce Hamp in both mice and humans. This increase in HAMP is thought to be mediated by the production of IL6 by macrophages, which then acts through the JAK-STAT pathway resulting in the production of HAMP (22, 23, 41). Mice lacking both Hfe and Tfr2 or Tfr2 alone have a reduced Hamp induction in response to inflammatory stimuli (36) as compared to WT mice, suggesting that TFR2 could play a role in inflammation-mediated Hamp induction.

In order to determine whether the absence of Tfr2 in macrophages affects inflammation-mediated iron homeostasis, control and KO mice were injected with
LPS (1 μg/g of body weight) or saline (control) for 6 hours and their tissues harvested for further analysis.

The mice treated with LPS for 6 hours had significantly lower serum iron levels as compared to controls (saline treated) (Figure 7A). These results are in agreement with previous studies which have shown that in the presence of infections or inflammatory stimuli serum iron levels decrease (23, 25). A decrease in transferrin saturation (Figure 7B) was observed in both the control and KO mice and no differences were observed between genotypes, suggesting that this was a LPS-specific effect and it did not require macrophage Tfr2 expression.

We investigated the role of macrophage TFR2 in the inflammation-mediated regulation of Hamp in the liver. As expected, the expression of Hamp increased significantly in the livers of mice treated with LPS (Figure 8A), and there were no significant differences between the control and KO mice. This suggests Tfr2 expression in macrophages is not required for the inflammation-mediated Hamp response in the liver.

The mRNA levels of Fpn1 and Hjv in the livers of the control and KO mice treated with LPS were significantly lower (Figure 8B and C). This is in accordance with several previously published studies which have reported the LPS-mediated down-regulation of Fpn1 and Hjv (17, 18, 26, 28, 42).

In order to determine whether macrophage-specific deletion of Tfr2 causes differences in the inflammatory response, we also measured the mRNA levels of orosomucoid 2 (Orm2) and serum amyloid A1 (Saa1) (Figure 8D and E), two known inflammatory markers expressed in the liver (36). The mRNA levels of these two genes were similar in the control and KO mice for both saline and LPS treatments.
Discussion

Previous studies have suggested that \textit{Tfr2} is expressed in macrophages \cite{15, 33}. The \(\beta\)-form of TFR2 was suggested to be involved in the transcription of \textit{Fpn1} specifically in macrophages \cite{33}. In order to examine the molecular functions of \textit{Tfr2} in macrophages, \textit{LysM-Cre} mice, which have been shown to express the Cre protein in cells of the macrophage lineage, including the peritoneal and splenic macrophages and Kupffer cells in the liver \cite{7, 27}, were crossed with \textit{Tfr2}\(^{f/f}\) mice generated in our laboratory \cite{40}. This resulted in the deletion of \textit{Tfr2} from the macrophages. Reduced expression of \textit{Tfr2} mRNA was detected specifically in peritoneal macrophages but not in the liver.

The loss of \textit{Tfr2} in the macrophages did not lead to any significant changes in the intracellular iron levels as we did not observe any differences in the mRNA levels of \textit{Tfr1}, \textit{Fth} or \textit{Ftl}. The mRNA levels of other genes known to be involved in macrophage iron homoeostasis did not differ in the peritoneal macrophages of the control and KO mice. These results suggested that loss of \textit{Tfr2} does not affect iron metabolism in the macrophages.

The levels of FPN mRNA and protein were significantly lower in the peritoneal macrophages of the KO mice supporting the previous observation that \textit{Tfr2} could be influencing \textit{Fpn1} mRNA levels \cite{32}. In order to test this we did a correlation between \textit{Tfr2} and \textit{Fpn1} expression in the peritoneal macrophages. The Pearson correlation coefficient was \(R= 0.9049\) and this correlation was also significant \((p=0.002)\).

Several factors including iron levels, inflammation and hypoxia have been shown to affect \textit{Fpn1} levels. We did not observe any significant differences in the mRNA levels of \textit{Tfr1}, \textit{Fth}, \textit{Ftl} which have been previously used as markers of intracellular iron.

This suggests that the decrease in \textit{Fpn1} is not due to altered iron levels in the
macrophages. Similarly we did not see any differences in the mRNA expression levels of the inflammatory markers Orm2 and Saa1, suggesting that the reduced Fpn1 levels are not due to increased basal inflammation. These results provide evidence that the reduced Fpn1 levels in the macrophages are due to loss of Tfr2 in the macrophages.

A reduction in FPN levels in the macrophages could lead to iron retention in these cells, which may in turn disrupt systemic iron homeostasis. In order to determine whether the reduced Fpn1 in macrophages of the KO mice leads to iron overload or dysregulated iron homoeostasis, we examined the role of macrophage Tfr2 in iron- and inflammation-mediated Hamp expression in the liver by either feeding the mice on an iron-rich diet or injecting them with LPS for 6 hours. The deletion of Tfr2 in macrophages did not affect systemic iron metabolism, suggesting that macrophage Tfr2 is not required for the systemic regulation of body iron levels. The expression levels of genes involved in regulating iron metabolism did not differ significantly in the livers of control and KO mice. Previously it has been suggested that β-Tfr2 is required for Fpn1 transcription in macrophages (32). The KO mice generated in our studies lack both α- and β- forms of Tfr2, whereas, the mice used in the study by Roetto et al. (32) lacked β-Tfr2 but expressed the α-form of Tfr2 in all other tissues. The macrophage-specific Tfr2-KO mice do not develop iron overload, similar to the mice used in the study by Roetto et al. (32), but since they lack both forms of Tfr2 in the macrophages this is a novel model to study the function of Tfr2 in the cells of macrophage lineage.
Acknowledgements

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Figure Legends

**Figure 1** Loss of Tfr2 in macrophages leads to a reduction in Fpn1 mRNA. mRNA expression levels of (A) Tfr2, (B) Fpn1, (C) Dmt1, (D) Hif1α, (E) Fth, (F) Ftl, (G) Sod2 and (H) Tfr1 (relative to the geometric mean of three reference genes: β-actin, Hprt and Polr2a) were measured in the peritoneal macrophages 5-week-old control (●Tfr2f/f) and KO (■Tfr2f/f/LysM-Cre+/−) male mice (n = 3-5 per group) fed a control. Data are shown as dot plots, showing the mean and standard error of the mean (SEM). Statistically significant differences (Student’s t-test; p<0.05) are denoted as (*) compared to the control genotype.

**Figure 2** Reduced FPN protein in the peritoneal macrophages of the KO mice. Immunoblotting was performed on protein lysates from the peritoneal macrophages of 5-week-old control (●Tfr2f/f) and KO (■Tfr2f/f/LysM-Cre+/−) male mice (n = 3 per group) fed a control diet and probed with antibodies against FPN and actin. Densitometric analysis of the protein bands were carried out for FPN (B) and measured relative to actin levels. The blots were quantitated using Genetools software version 4. The graph B shows the ratio between FPN and actin. Data are shown as dot plots, showing the mean and SEM. Statistically significant differences (Student’s t test; p<0.05) are denoted as (b) compared to the respective control genotype.

**Figure 3** Iron parameters in the control and KO mice. (A) Total serum iron, (B) transferrin saturation, (C) hepatic iron concentration (HIC) and (D) splenic iron concentration (SIC) were measured in the spleen and liver of 5-week-old control (●Tfr2f/f) and KO (■Tfr2f/f/LysM-Cre+/−) male mice (n = 5 per group) fed a control
(Control diet) or an iron-rich diet (High-Fe diet). Data are shown as dot plots, showing the mean and SEM. Statistically significant differences (two-way ANOVA using Tukey’s multiple comparison test; p<0.05) are denoted as (b) compared to the respective control treatment group.

**Figure 4** Perls’ staining of liver and spleen sections. Perls’ staining was performed on liver and spleen sections from representative 5-week-old control and KO male mice fed a control (Control diet) or an iron-rich diet (High-Fe diet). Scale bar = 200 µm.

**Figure 5** Expression of iron metabolism genes in the livers of control and KO mice fed a control or an iron-rich diet. mRNA expression levels of (A) Bmp6, (B) Hamp, (C) Id1, (D) Smad7, (E) Hfe, (F) Hjv, (G) Fpn1 and (H) Tfr2 (relative to the geometric mean of three reference genes: β-actin, Hprt and Polr2a) were measured in the livers of 5-week-old control (● Tfr2f/f) and KO (■ Tfr2f/f/LysM-Cre+/−) male mice (n = 5 per group) fed a control (Control diet) or an iron-rich diet (High-Fe diet). Data are shown as dot plots, showing the mean and SEM. Statistically significant differences (two-way ANOVA using Tukey’s multiple comparison test; p<0.05) are denoted as (a) compared to the control genotype and (b) compared to the respective control treatment group.

**Figure 6** An iron-rich diet increases phosphorylation of SMAD1/5 in the livers of the control and KO mice. Immunoblotting was performed using 25µg of total liver homogenates from 5-week-old control and KO male mice fed a control (Control diet) or an iron-rich diet (High-Fe diet) (n = 5 per group) and probed with antibodies
against TFR2, pSMAD1/5, and Actin. (A) Representative image of a blot performed at least three times and on all five mice from each group. Densitometric analysis of the protein bands were carried out for TFR2 (B) and pSMAD1/5 (C) and measured relative to Actin levels. The blots were quantitated using Genetools software version 4. The graphs show the ratios between TFR2 or pSMAD1/5 and Actin. Data are shown as dot plots, for control (● Tfr2/−/−) and KO (■ Tfr2/−/−/LysM-Cre+/−) mice showing the mean and SEM (n = 5 per group). Statistically significant differences (two-way ANOVA with Tukey’s multiple comparison test; p<0.05) are denoted as (b) compared to the respective control treatment group.

Figure 7 Iron parameters in the control and KO mice treated with saline or LPS. (A) Total serum iron, (B) transferrin saturation, (C) hepatic iron concentration (HIC) and (D) splenic iron concentration (SIC) were measured in the spleen and liver of 5-week-old control (● Tfr2/−/−) and KO (■ Tfr2/−/−/LysM-Cre+/−) male mice (n = 5 per group) treated with saline or LPS for 6 hours. Data are shown as dot plots, showing the mean and SEM. Statistically significant differences (two-way ANOVA using Tukey’s multiple comparison test; p<0.05) are denoted as (b) compared to the respective control treatment group.

Figure 8 Expression of genes in the livers of control and KO mice treated with saline or LPS. mRNA expression levels of (A) Hamp, (B) Fpn1, (C) Hjv, (D) Orm2 and (E) Saa1 (relative to the geometric mean of three reference genes: β-actin, Hprt and Polr2a) were measured in the livers of 5-week-old control (● Tfr2/−/−) and KO (■ Tfr2/−/−/LysM-Cre+/−) male mice (n = 5 per group) treated with saline or LPS for 6 hours. Data are shown as dot plots, showing the mean and SEM. Statistically
significant differences (two-way ANOVA using Tukey’s multiple comparison test; p<0.05) are denoted as (b) compared to the respective control treatment group.
### Table 1. Primers used in the study

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Figure 2 Rishi et al
Figure 4. Rishi et al.
Figure 5 Rishi et al
Figure 6. Rishi et al
Figure 7. Rishi et al