Metalloreductase Steap3 coordinates the regulation of iron homeostasis and inflammatory responses

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ABSTRACT

Background
Iron and its homeostasis are intimately related to inflammatory responses, but the underlying molecular mechanisms are poorly understood. We investigated the role of Steap3 in regulating iron homeostasis in macrophages, and the effects of Steap3 depletion on host inflammatory responses.

Design and Methods
We analyzed bone marrow-derived macrophages and primary cultured hepatocytes from *Steap3*$^{-/-}$ mouse models to investigate the roles of Steap3 in coordinately regulating iron homeostasis and inflammatory responses. First, we examined iron distribution and iron status in cells deficient in Steap3, as well as the requirement for the *Steap3* gene during inflammatory responses. Secondly, we analyzed the regulation of *Steap3* expression by inflammatory stimuli and thus, the influence of these stimuli on iron distribution and homeostasis.

Results
We found that *Steap3* mRNA was expressed at high levels in macrophages and hepatocytes. Steap3 deficiency led to impaired iron homeostasis, causing abnormal iron distribution and a decreased availability of cytosolic iron in macrophages. Among STEAP family members, *Steap3* mRNA was uniquely down-regulated in macrophages stimulated by lipopolysaccharides. To determine whether Steap3 regulated iron homeostasis during inflammatory stress, we treated *Steap3*$^{-/-}$ mice with lipopolysaccharide, which produced greater iron accumulation in the vital tissues of these mice compared to in the tissues of wild-type controls. Furthermore, Steap3 depletion led to impaired induction of interferon-β, monocyte chemoattractant protein-5, and interferon induced protein-10 in macrophages via the TLR4-mediated signaling pathway.

Conclusions
Steap3 is important in regulating both iron homeostasis and TLR4-mediated inflammatory responses in macrophages. Steap3 deficiency causes abnormal iron status and homeostasis, which leads to impaired TLR4-mediated inflammatory responses in macrophages. Following inflammatory stimuli, Steap3 depletion causes dysregulated iron sequestration and distribution. Our results provide important insights into the function of Steap3 as a coordinate regulator of both iron homeostasis and innate immunity.

Key words: Steap3, iron, macrophage, inflammation.

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The online version of this article has a Supplementary Appendix.

Introduction

In eukaryotes, iron exists in two valence states, ferrous (Fe^{2+}) and ferric (Fe^{3+}). Fe^{3+} can bind to transferrin, the major carrier of iron in the circulation and extravascular fluid, to form holo-transferrin, which is taken up by receptor-mediated endocytosis of transferrin receptor on the cell membrane. The endosome containing transferrin-trasnferrin receptor-1 complex is then acidified, which facilitates release of ferric iron from holo-transferrin.[1] However, ferric iron must be reduced to the ferrous form before it can be transported out to the cytoplasm by the divalent metal transporter-1 (DMT1)[2,3] or transient receptor potential (TRP) protein TRPML1.[4]

Six-transmembrane epithelial antigen of the prostate 3 (Steap3) was recently characterized as a ferrireductase that reduces ferric iron to ferrous iron in endosomes.[5,6] Steap3 belongs to the STEAP family, which bears homology to oxidoreductases found in bacteria and archaea as well as the FRE metalloreductase in yeast.[5,6] There are four members in the STEAP family, Steap1, Steap2, Steap3, and Steap4.[5] Although the four members of the STEAP family have different expression profiles, they are all localized to the plasma membrane and/or endosomes. In addition, all Steap proteins except Steap1 demonstrate ferrireductase activity when over-expressed in human embryonic kidney cells.[5] *Steap3* was initially described as a putative tumor suppressor gene capable of inhibiting tumor cells through a caspase-3 dependent pathway.[7-9] It was subsequently reported to facilitate exosome secretion via a non-classic pathway.[10,11] Previous work in mouse models showed that a lack of *Steap3* leads to a hypochromic, microcytic anemia and disrupts iron metabolism; this results in iron overload in serum and tissues.[12] Recently, a nonsense mutation in the *Steap3* gene was found to be associated with a novel type of congenital hypochromic anemia.[13]

Here we demonstrate that Steap3 is the only STEAP family member highly expressed in macrophages, a cell population that functions in innate immunity. The functional characteristics illustrate the crucial roles played by macrophages in both modulation of iron homeostasis and immune responses. During infections, available free iron is decreased and becomes sequestered in cells of the reticuloendothelial system, especially macrophages, due to inflammatory signaling cascades.[14,15] Iron is required by host cells for normal cellular function and immune-mediated defense mechanisms. For example, iron homeostasis can affect the macrophage effector functions that influence downstream innate and adaptive immune responses.[15,16]

The interactions between iron status and immune function are well-described but poorly understood, particularly with regard to the molecular mechanisms that regulate these interactions. This study was conducted to investigate such interactions.

Design and Methods

The design and methods of this study are described in full in the *Online Supplementary Design and Methods*.[5,6,17] Briefly, we used *Steap3*$^{-/-}$ mice and control wild-type *Steap3*$^{+/+}$ mice provided with a standard rodent laboratory diet, injected intraperitoneally with a single dose of lipopolysaccharide (LPS) or the same volume of phosphate-buffered saline as a control. Blood and tissues were harvested for protein, iron and gene expression assays at various time points.

Statistical analysis

Data are presented as mean ± SEM. All experiments were performed in triplicate. Student's t-test was used for comparison between two groups. P values < 0.05 were considered statistically significant.

Results

Steap3 is a ferrireductase and is highly expressed in macrophages and hepatocytes

Macrophages and hepatocytes are both important regulators of iron storage and metabolism. To investigate the roles played by the indispensable iron reductases, which control iron metabolism in these two cell types, we analyzed the expression of STEAP mRNA. Doing so revealed that the expression of *Steap3* was over 200-fold higher in bone marrow derived macrophages (BMDMs) than *Steap1*, *Steap2*, and *Steap4* in wild-type mice (Figure 1A). In primary cultured mouse hepatocytes, *Steap3* was again the most highly expressed, while the other STEAP family members were expressed at similar levels to each other, and was expressed to a greater extent in these cells than in BMDMs (Figure 1B). We next examined *Steap3*$^{-/-}$ macrophages and hepatocytes to determine whether mRNA levels of the three other STEAP family members were altered, which would suggest possible compensatory regulation. The mRNA for *Steap2* and *Steap4*, which have ferrireductase activity, was consistently and significantly up-regulated in macrophages in the absence of *Steap3* (Figure 1C), however, the expression of *Steap2* and *Steap4* mRNA still did not reach the level of *Steap3* expression in wild-type BMDMs. In *Steap3*$^{-/-}$ hepatocytes, *Steap2* was significantly up-regulated, whereas *Steap1* and *Steap4* mRNA levels were unchanged (Figure 1D). Since *Steap3* was the only highly expressed STEAP member in macrophages, this suggests that *Steap3* might play an important role in these cells, which are responsible for both maintaining iron homeostasis and regulating inflammatory immune responses. Thus, we measured ferrireductase activity in BMDMs and hepatocytes from *Steap3*$^{-/-}$ *versus* control mice, and found significant reductions in both the knockout BMDMs (Figure 1E) and the hepatocytes (Figure 1F).

Abnormal iron status in Steap3$^{-/-}$ *macrophages and hepatocytes*

As *Steap3* was the only STEAP reductase family member that was highly expressed in macrophages, we investigated whether *Steap3* depletion affects iron homeostasis and metabolism in macrophages. First, we analyzed the expression of important cellular iron transporters and regulators such as *Hfe*, *HO-1*, *Fpn1* and *Dmt1* in *Steap3*$^{-/-}$ BMDMs, because altered expression might influence net iron homeostasis. All four mRNA were similarly expressed in *Steap3*$^{-/-}$ and wild-type BMDMs (*Online Supplementary Figure S1A*). We then investigated whether *Steap3* deficiency led to disturbances in macrophage iron homeostasis as indicated by iron distribution and content. Using scanning transmission X-ray microscopy spectromicroscopy analysis, we found that iron was present in a

more concentrated distribution in *Steap3*$^{-/-}$ macrophages (compacted, pseudo-colored red plaques in *Online Supplementary Figure S1B*, right panel), than in *Steap3*$^{+/+}$ macrophages (disperse, pseudo-colored red regions in *Online Supplementary Figure S1B*, left panel). The total iron content within whole cells was measured by inductively coupled plasma mass spectrometry. The results showed that total iron content was similar between *Steap3*$^{+/+}$ and *Steap3*$^{-/-}$ BMDMs, before or after treatment with ferric ammonium citrate (FAC) (Figure 2A). The cytochemical calcein-AM (calcein- acetoxymethyl ester) method is an established technique for assaying the 'labile iron pool' of cells.[18,19] The calcein assay showed higher fluorescence in *Steap3*$^{-/-}$ BMDMs, indicating lower cytosolic iron (Figure 2B). Similar results were obtained when examining the protein levels of ferritin-H and ferritin-L, both of which are post-transcriptionally controlled by low cytosolic iron.[20] Both before and after FAC treatment, ferritin levels were lower in *Steap3*$^{-/-}$ BMDMs than in wild-type cells (Figure 2C). Collectively, these data suggest that *Steap3*$^{-/-}$ mice could not reduce Fe^{3+} to Fe^{2+} for release into the cytoplasm, which caused unbalanced iron distribution as iron was congregated in some compartments, but also displayed lower available cytosolic iron, although the total cellular iron content was similar to that in the wild-type animals. Additionally, we performed the experiments in primary cultured hepatocytes. The results showed that the total iron content was higher in *Steap3*$^{-/-}$ hepatocytes than in *Steap3*$^{+/+}$ hepatocytes (Figure 2D). However, the calcein assay and ferritin protein levels all suggested similar cytosolic iron levels in these two hepatocyte populations (Figure 2E and 2F). We then analyzed the iron status in liver and spleen. The iron content of liver and spleen was higher in *Steap3*$^{-/-}$ mice than in wild-type mice (Figure 2G). However, in the liver, ferritin-H and ferritin-L levels were the same in both *Steap3*$^{+/+}$ and *Steap3*$^{-/-}$ mice, whereas in the spleen, ferritin-H and ferritin-L levels were both lower in *Steap3*$^{-/-}$ mice than in *Steap3*$^{+/+}$ ones (Figure 2H). Together, these results confirm that deficiency in Steap3 causes abnormal cellular iron status.

Steap3 *is down-regulated by lipopolysaccharide in macrophages*

Since macrophages play important roles in innate immune function, we investigated whether *Steap3* contributed functionally to macrophage-mediated innate immunity. First, we determined whether *Steap3* could be regulated by an infectious stimulus. We used LPS, an agonist for TLR4, to treat wild-type BMDMs for 24 h, and examined the relative mRNA levels of STEAP family members. Doing so revealed that *Steap3* mRNA declined markedly after LPS treatment (Figure 3A). In contrast, *Steap4* was up-regulated by LPS at a very early time point, consistent with reports that *Steap4* could be up-regulated by tumor necrosis factor-alpha (TNF-α.).[21] *Steap1* and *Steap2* were also transiently increased after LPS stimulation (*Online Supplementary Figure S2A*).

To determine whether the down-regulation of *Steap3* mRNA after LPS administration also existed in other types of cells, we treated wild-type hepatocytes with LPS as above. Surprisingly, *Steap3* mRNA did not change with LPS treatment in hepatocytes (Figure 3B). However, we did find that *Steap1*, *Steap2*, and *Steap4* were up-regulated by LPS, indicating a similar regulation pattern as in macrophages (*Online Supplementary Figure S2B*).

One possible explanation for the above results was that LPS-stimulated macrophages, but not hepatocytes, secreted unique factors[22] that led to *Steap3* mRNA down-regulation. To test this hypothesis, we incubated wild-type hepatocytes with culture medium from wild-type BMDMs that had been plated with or without LPS stimulation. When hepatocytes were cultured overnight in conditioned medium derived from macrophages plated without LPS stimulation, *Steap3* mRNA remained unchanged in hepatocytes (Figure 3C). However, when hepatocytes were incubated with conditioned medium derived from macrophages stimulated overnight with LPS, we observed a striking down-regulation of *Steap3* mRNA (Figure 3D). The same pattern of down-regulation was also found in primary cultured peritoneal macrophages (Figure 3E), as well as bulk bone marrow (Figure 3F), and liver preparations (Figure 3G) from LPS-stimulated mice. These results suggested that transcriptional regulation of *Steap3* or mRNA stability after an inflammatory stimulus was different from that of other STEAP family members, and that *Steap3* mRNA could be down-regulated by factors secreted by macrophages after LPS stimulation. As demonstrated above, Steap3 deficiency caused iron retention in cells, but simultaneously led to low availability of cytosolic iron. Collectively, these facts imply that down-regulation of *Steap3* mRNA might contribute to the sequestration of iron in macrophages during inflammatory stimulation.

Systemic and cellular iron homeostasis are disturbed in Steap3$^{-/-}$ *mice following lipopolysaccharide stimulation*

To find out whether Steap3 played a part in the redistribution and sequestration of iron in mice during inflammatory conditions, we treated wild-type (8-week old, male) and *Steap3*$^{-/-}$ mice (8-week old, male) with LPS at a dose of 5 mg/kg for 3 or 6 h. In wild-type mice, serum iron and transferrin saturation were decreased after LPS treatment, and were accompanied by increased unsaturated iron-binding capacity (Figure 4A-4D), indicative of a link between iron and inflammatory responses known as anemia of inflammation.[23] However, in *Steap3*$^{-/-}$ mice treated with LPS, serum iron concentrations, total iron-binding capacity and transferrin saturation only showed a marginally decreasing trend (P=0.08) at 6 h after LPS treatment (Figure 4A-4D).

As potential indicators of dysregulated iron homeostasis, we next examined iron content in livers and spleens of *Steap3*$^{-/-}$ mice. In *Steap3*$^{-/-}$ mice, the liver iron content after LPS stimulation was markedly increased compared to that in wild-type mice, (Figure 4E). In *Steap3*$^{-/-}$ mice, splenomegaly was present even before LPS treatment, and spleens remained enlarged after LPS stimulation (Figure 4F). The splenic iron concentrations in *Steap3*$^{-/-}$ mice were higher than those in control mice (Figure 4G). The total iron content in *Steap3*$^{-/-}$ spleens was also greater than that present in control mice, both before and after LPS treatment (Figure 4H). As an important hormonal regulator of systemic iron homeostasis, we examined hepcidin mRNA expression, which is encoded by *Hamp1*, in wild-type and Steap3-deficient mice before and after LPS stimulation. Whereas wild-type mice showed a significant increase in *Hamp1* mRNA at 3 h post-LPS stimulation, *Steap3*$^{-/-}$ mice showed a relatively mild increase in hepcidin (Figure 4I). This observation might account for the uniform serum iron concentrations observed in knock-out mice (Figure 4A), since low hepcidin expression could cause increased

iron uptake in the duodenum via enhanced expression of *Fpn1*.[1] We also investigated the serum concentrations of pro-inflammatory cytokines TNF-α and interleukin 6 (IL-6), both of which were significantly higher in *Steap3*$^{-/-}$ mice treated with LPS for 3 h than in control mice (Figure 4J, 4K). Additionally, we examined the mRNA level of TNF-α and IL-6 in livers since hepatic inflammatory responses are also physiologically important. The levels of TNF-α (*Online Supplementary Figure S3A*) were the same in both wild-type and *Steap3*$^{-/-}$ mice, while the concentration of IL-6 (*Online Supplementary Figure S3B*) was slightly higher in *Steap3*$^{-/-}$ mice than in wild-type ones. These results indicate that Steap3 depletion could affect the redistribution of iron in serum and tissues and support our hypothesis that Steap3 might function in regulating infection-induced sequestration of iron.

TLR4-mediated inflammatory responses are impaired in Steap3$^{-/-}$ *macrophages*

Since we demonstrated that inflammatory stimuli could alter iron distribution in a manner that was partially due to Steap3, we then investigated how abnormal iron status in *Steap3*$^{-/-}$ mice could affect the inflammatory responses in macrophages. We treated BMDMs from wild-type and *Steap3*$^{-/-}$ mice with LPS and analyzed the mRNA expression of several pro-inflammatory factors over time. Following LPS administration, *TNF-α* (Figure 5A) and *IL-6* (Figure 5B) were up-regulated to similar levels in *Steap3*$^{-/-}$ macrophages and their wild-type counterparts. *IFN-β* was dramatically diminished in *Steap3*$^{-/-}$ macrophages following LPS stimulation (Figure 5C). The phosphorylation of STAT1 downstream of *IFN-β* was consistently impaired after LPS stimulation in *Steap3*$^{-/-}$ macrophages (Figure 5G). Not surprisingly, the expression of *MCP-5* (Figure 5D), *IP-10* (Figure 5E) and *RANTES* (Figure 5F) was decreased as well, since they function downstream of IFN-β. IFN-β can be induced by LPS via a TLR4-mediated, Myd88-dependent pathway, or by poly(I:C) via a TLR3-mediated, Myd88-independent pathway.[24,25] We treated BMDMs with the TLR2 agonist Pam3CSK4 or the TLR3 agonist poly(I:C), which induce inflammatory responses through Myd88-dependent and Myd88-independent pathways, respectively,[25,26] to identify which *IFN-β*-inducing pathway was impaired in *Steap3*$^{-/-}$ macrophages. As expected, Pam3CSK4 could not induce *IFN-β*, *MCP-5*, or *IP-10*. However, following poly(I:C) treatment, which could trigger the TLR3-mediated, Myd88-independent pathway, there were no differences in the expression of *TNF-α*, *IL-6*, *IFN-β*, *MCP-5*, *IP-10*, or *RANTES* between *Steap3*$^{+/+}$ or *Steap3*$^{-/-}$ macrophages (Figure 5A-5F). Of note, the poly (I:C)-induced phosphorylation of STAT1 was also equivalent between wild-type and knock-out macrophages (Figure 5G). Despite the fact that poly(I:C) and LPS could both induce *IFN-β* through a Myd88-independent pathway using the same adaptor protein, TIR-domain-containing adapter inducing interferon-β (TRIF), TLR3 and TLR4 have different adaptors for signal transduction. MyD88-independent *IFN-β* production and activation of signaling cascades are TRIF-related adaptor molecule (TRAM)-dependent in TLR4-mediated signaling, while they are TRAM-independent in TLR3-mediated induction.[27] Our results show that depletion of *Steap3* might

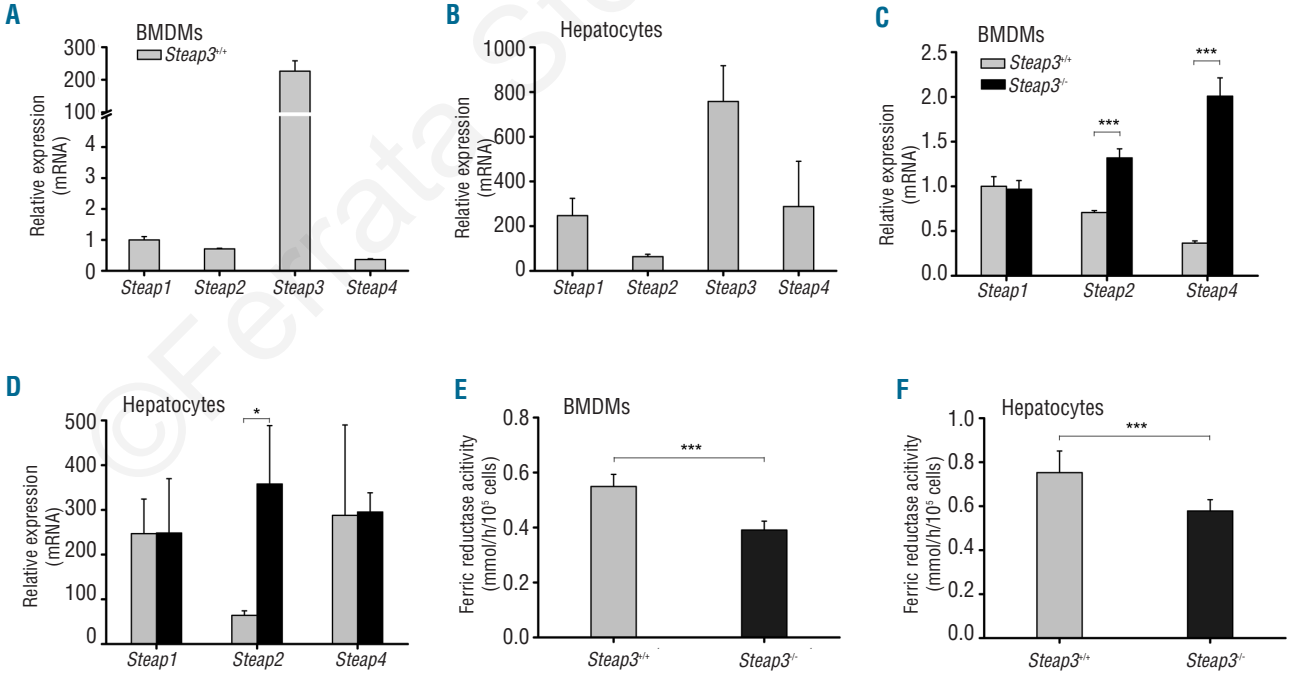

Figure 1. ***Steap*** mRNA levels and ferrireductase activities in ***Steap3***$^{-/-}$ macrophages and hepatocytes. Relative mRNA levels of ***Steap1***, ***Steap2***, ***Steap3*** and ***Steap4*** were measured by qRT-PCR in (A) bone marrow-derived macrophages (BMDMs), and (B) primary cultured hepatocytes from C57BL/6J wild-type mice (male, 8-10 weeks, n=4). The mRNA expression of ***Steap1***, ***Steap2***, ***Steap4*** in BMDMs (C) and in hepatocytes (D) from ***Steap3***$^{+/+}$(male, 8-10 weeks, n=4) ***versus*** ***Steap3***$^{-/-}$ mice (male, 8-10 weeks, n=4). Relative RNA abundance is normalized to ***Steap1*** in macrophages, which was defined as a ratio of 1.0. Ferrireductase activity was analyzed in BMDMs (E) and hepatocytes (F). Data represent mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001.

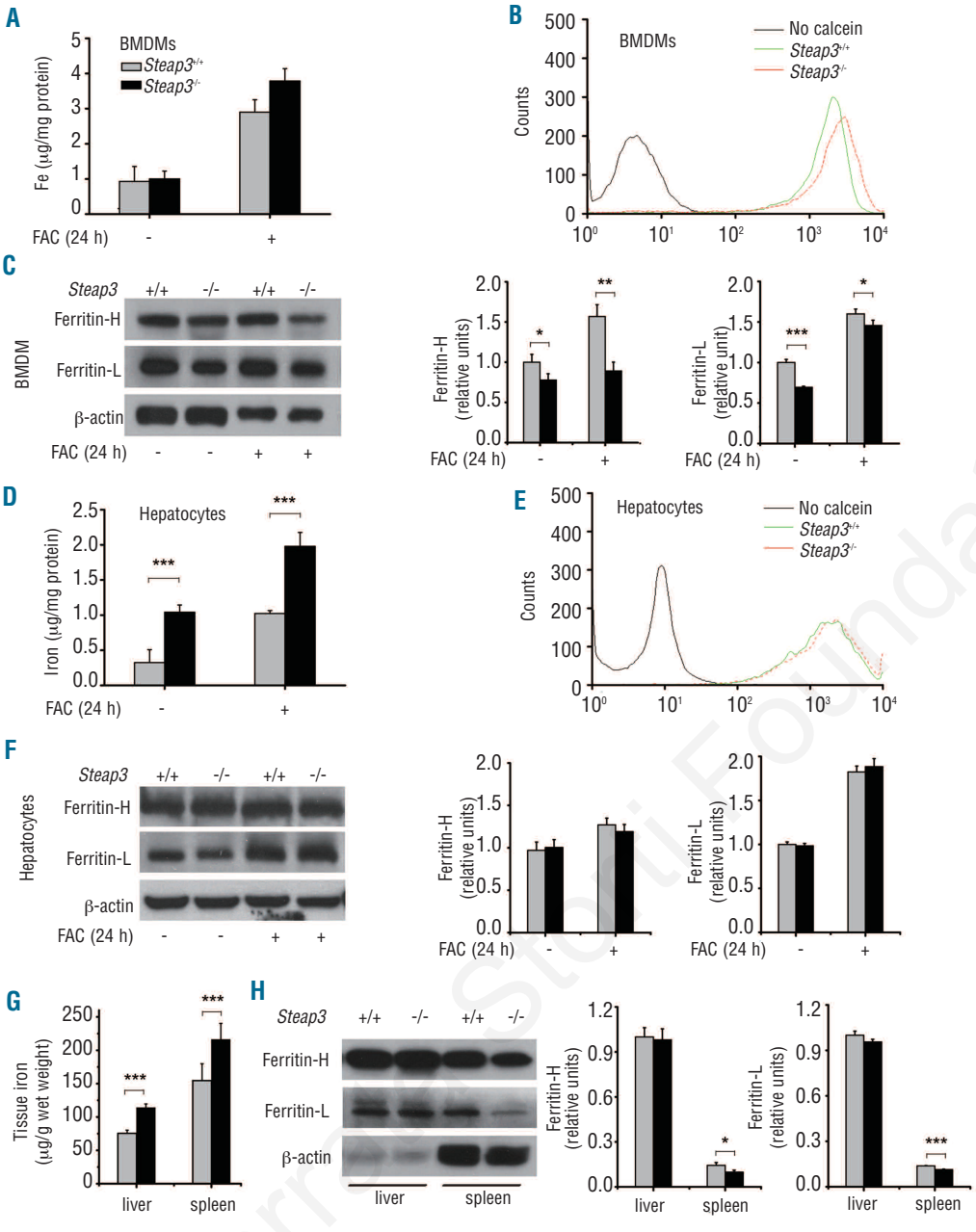

Figure 2. Abnormal iron status in *Steap3*$^{-/-}$ macrophages and hepatocytes. Inductively coupled plasma mass spectrometry analysis of iron content in BMDMs (n≥5) (A) and hepatocytes (D) that were cultured in medium with or without FAC (100 mM) for 24 h. Black bars = *Steap3*$^{-/-}$, gray bars = *Steap3*$^{+/+}$. Calcein fluorescence histograms of *Steap3*$^{+/+}$ and *Steap3*$^{-/-}$ BMDM (B) and hepatocytes (E). The black histogram depicts fluorescence of wild-type cells in the absence of calcein. The green histogram depicts the calcein fluorescence of wild-type cells and the red histogram represents the calcein fluorescence of *Steap3*$^{-/-}$ cells. Ferritin-H and ferritin-L protein levels of BMDMs (C) and hepatocytes (F), treated with or without FAC (100 mM), from *Steap3*$^{+/+}$ and *Steap3*$^{-/-}$ mice were assessed by western blot analysis. Ferritin-H/actin and ferritin-L/actin ratios were quantitated by densitometry in three independent experiments. (G) Liver and spleen iron content of *Steap3*$^{+/+}$ and *Steap3*$^{-/-}$ mice. (H) Ferritin-H and ferritin-L protein levels of liver and spleen in *Steap3*$^{+/+}$ and *Steap3*$^{-/-}$ mice were assessed by western blot analysis and ferritin-H/actin and ferritin-L/actin ratios were quantitated by densitometry in three independent experiments. Data represented are the mean ± SEM. Similar results were obtained in three separate experiments. **P*<0.05, ***P*<0.01, ****P*<0.001.

specifically impair the TLR4-mediated, Myd88-independent pathway. This finding further confirmed the function of Steap3 as a coordinate regulator of both iron metabolism and inflammatory responses.

Deletion of Steap3 *inhibited iron-enhanced TLR4-mediated inflammatory responses*

Our results to this point showed an impaired TLR4-mediated, TRIF/TRAM-dependent inflammatory response and abnormal iron status, indicated by low availability of cytosolic iron in *Steap3*$^{-/-}$ macrophages. To confirm the role of iron in this TLR4-mediated pathway, we treated wild-type and *Steap3*$^{-/-}$ macrophages with FAC. FAC treatment significantly enhanced TLR4-mediated inflammatory responses, evidenced by increased expression of *IFN-β*, *MCP-5*, and *IP-10* (Figure 6A-6C), and increased phosphorylation of STAT1 (Figure 6D). Consistent with the above results, these cytokines were decreased in *Steap3*$^{-/-}$ mice with or without FAC treatment, as was the phosphorylation of STAT1. These results further confirmed that low cytosolic available iron in *Steap3*$^{-/-}$ macrophages could impair TLR4-mediated cellular inflammatory responses.

Discussion

Iron and its homeostasis are intimately related to inflammatory responses and, therefore, provide major protective mechanisms in human physiology. However, the molecular basis for the interaction between iron and immune function is not well understood. In the past decade, a large number of experimental studies showed that responses to inflammation and susceptibility to infection worsen with iron overload. Hereditary hemochromatosis with iron overload has been reported to confer

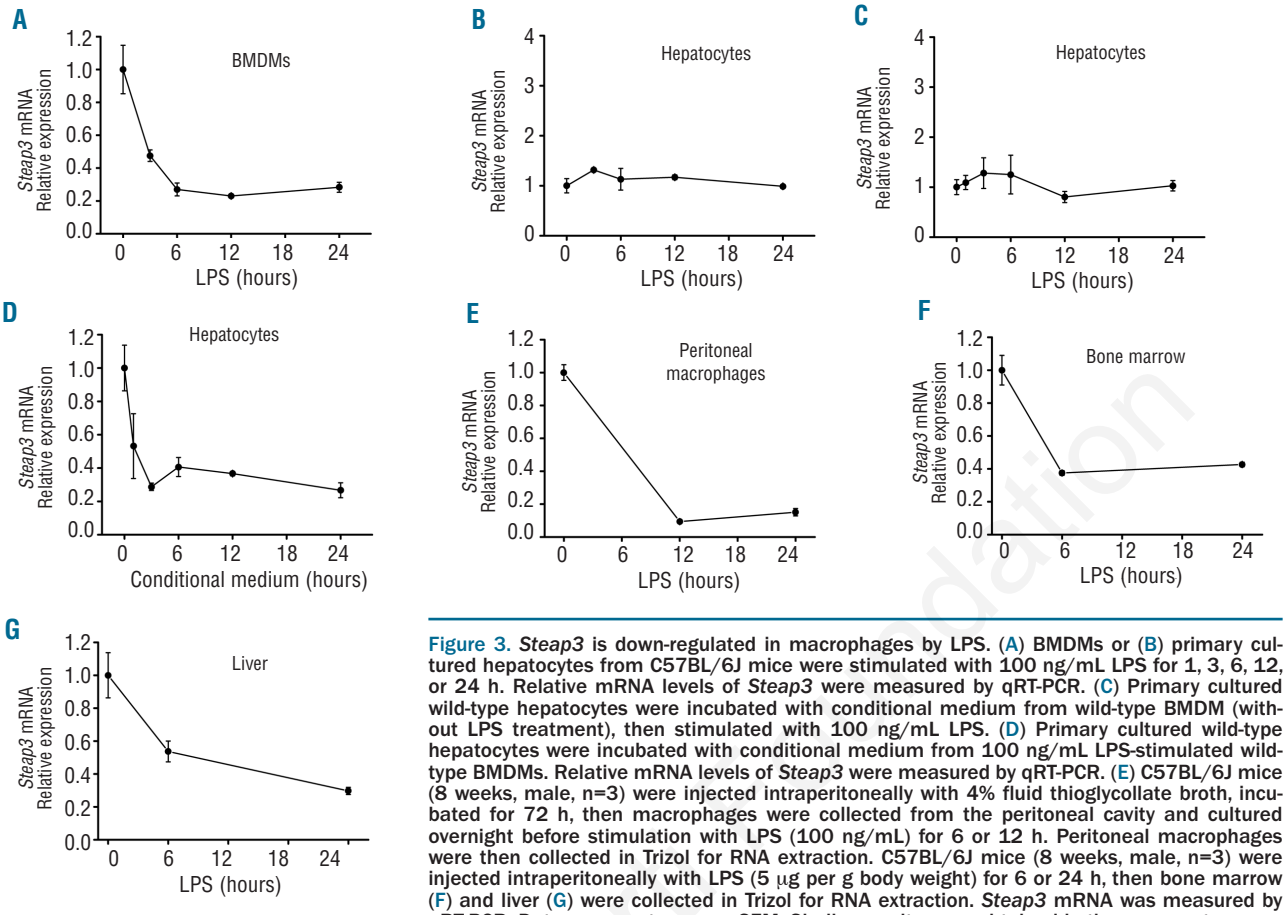

Figure 3. ***Steap3*** **is down-regulated in macrophages by LPS. (A) BMDMs or (B) primary cultured hepatocytes from C57BL/6J mice were stimulated with 100 ng/mL LPS for 1, 3, 6, 12, or 24 h. Relative mRNA levels of** ***Steap3*** **were measured by qRT-PCR. (C) Primary cultured wild-type hepatocytes were incubated with conditional medium from wild-type BMDM (without LPS treatment), then stimulated with 100 ng/mL LPS. (D) Primary cultured wild-type hepatocytes were incubated with conditional medium from 100 ng/mL LPS-stimulated wild-type BMDMs. Relative mRNA levels of** ***Steap3*** **were measured by qRT-PCR. (E) C57BL/6J mice (8 weeks, male, n=3) were injected intraperitoneally with 4% fluid thioglycollate broth, incubated for 72 h, then macrophages were collected from the peritoneal cavity and cultured overnight before stimulation with LPS (100 ng/mL) for 6 or 12 h. Peritoneal macrophages were then collected in Trizol for RNA extraction. C57BL/6J mice (8 weeks, male, n=3) were injected intraperitoneally with LPS (5 μg per g body weight) for 6 or 24 h, then bone marrow (F) and liver (G) were collected in Trizol for RNA extraction.** ***Steap3*** **mRNA was measured by qRT-PCR. Data represent mean ± SEM. Similar results were obtained in three separate experiments.**

susceptibility to infectious pathogens, while iron deficiency confers relative resistance to infection.[28-30] Here we demonstrated that the ferrireductase Steap3 is a coordinate regulator of inflammatory responses and iron homeostasis.

In the current study, we found that *Steap3* was the only STEAP family member that is highly expressed in macrophages, an important type of cell that links iron homeostasis and inflammatory responses. Furthermore, we demonstrated that Steap3 acts as a ferrireductase in macrophages and hepatocytes. Conceivably, in Steap3-depleted cells, ferric iron could not be reduced to ferrous iron, which is the only form able to be transported to the cytoplasm. Thus, iron might be accumulated in endosomes and lysosomes, accompanied by decreased cytosolic iron. As expected, we found that available cytosolic iron was lower in *Steap3*$^{-/-}$ BMDMs than wild-type BMDMs, although both strains had similar cellular iron content. *Steap3*$^{-/-}$ hepatocytes had higher total cellular iron but the same cytosolic iron compared to wild-type hepatocytes, which might be due to the compensatory effects of the relatively high expression of *Steap2* and *Steap4*. Notably, after FAC treatment, both *Steap3*$^{-/-}$ BMDMs and hepatocytes could still increase cytosolic iron even though the transport mechanism from endosome to cytosol was blocked. This might be due to involvement of other pathways of iron uptake, such as non-transferrin bound iron, which does not go through the endosome reducing pathway. We sought to understand the function of *Steap3* in the regulation of iron homeostasis and inflammatory responses by focusing initially on two aspects. First, we determined how an inflammatory stimulus altered iron status and sequestration of iron. Second, we examined how the disturbance of iron status affected inflammatory responses.

To answer the first question, we found a specific down-regulation of Steap3 mRNA after LPS stimulation, which was the opposite of the transient spikes in other Steap mRNA. Furthermore, while the up-regulation by LPS of other STEAP family members was also observed in hepatocytes, *Steap3* expression was not changed in these cells. This observation implied that secreted factors from macrophages are required for down-regulation of *Steap3* under inflammatory conditions. We then confirmed this hypothesis by incubating hepatocytes with LPS-treated conditioned medium from BMDMs, which resulted in down-regulation of *Steap3*. Identification of the specific soluble factors secreted from macrophages which cause *Steap3* down-regulation will require further study. TNF-α and IL-6 are not likely to be the regulators because, like macrophages, hepatocytes can also secrete TNF-α and IL-6 (*data not shown*).

Combined with the fact that iron was retained in *Steap3*-deficient cells, these results implied that down-reg-

ulation of *Steap3* might be a requisite for the sequestration of iron during inflammatory stimulation. This was further confirmed in LPS-challenged mice. We found abnormal iron distribution in serum and tissues of *Steap3*$^{-/-}$ mice after LPS stimulation compared to the distribution in wild-type controls. *Steap3*$^{-/-}$ mice expressed less liver hepcidin than wild-type mice after LPS stimulation, which could cause high expression of Fpn1 in the duodenum leading to enhanced uptake of iron. This might partially account for the unchanged serum iron and transferrin saturation in *Steap3*$^{-/-}$ mice. These findings would complement other known iron sequestration mechanisms through post-translational down-regulation of FPN1, mediated by the increased circulating levels of hepcidin associated with infection,[31] and mechanisms mediated by Nramp1, lactoferrin, and siderocalin, which have well-described functions in innate immunity.[32-35]

With regards to the second question, we hypothesized that depletion of Steap3 might cause abnormal inflammatory responses in macrophages. To confirm this, we used LPS to treat BMDM. The results showed that *IFN-β* induction in *Steap3*$^{-/-}$ macrophages was severely impaired compared to that in controls. LPS-induced up-regulation of *IFN-β* was mediated by TRAM/TRIF signals through a TLR4-mediated, Myd88-independent signal transduction pathway.[36,37] There was no significant difference between

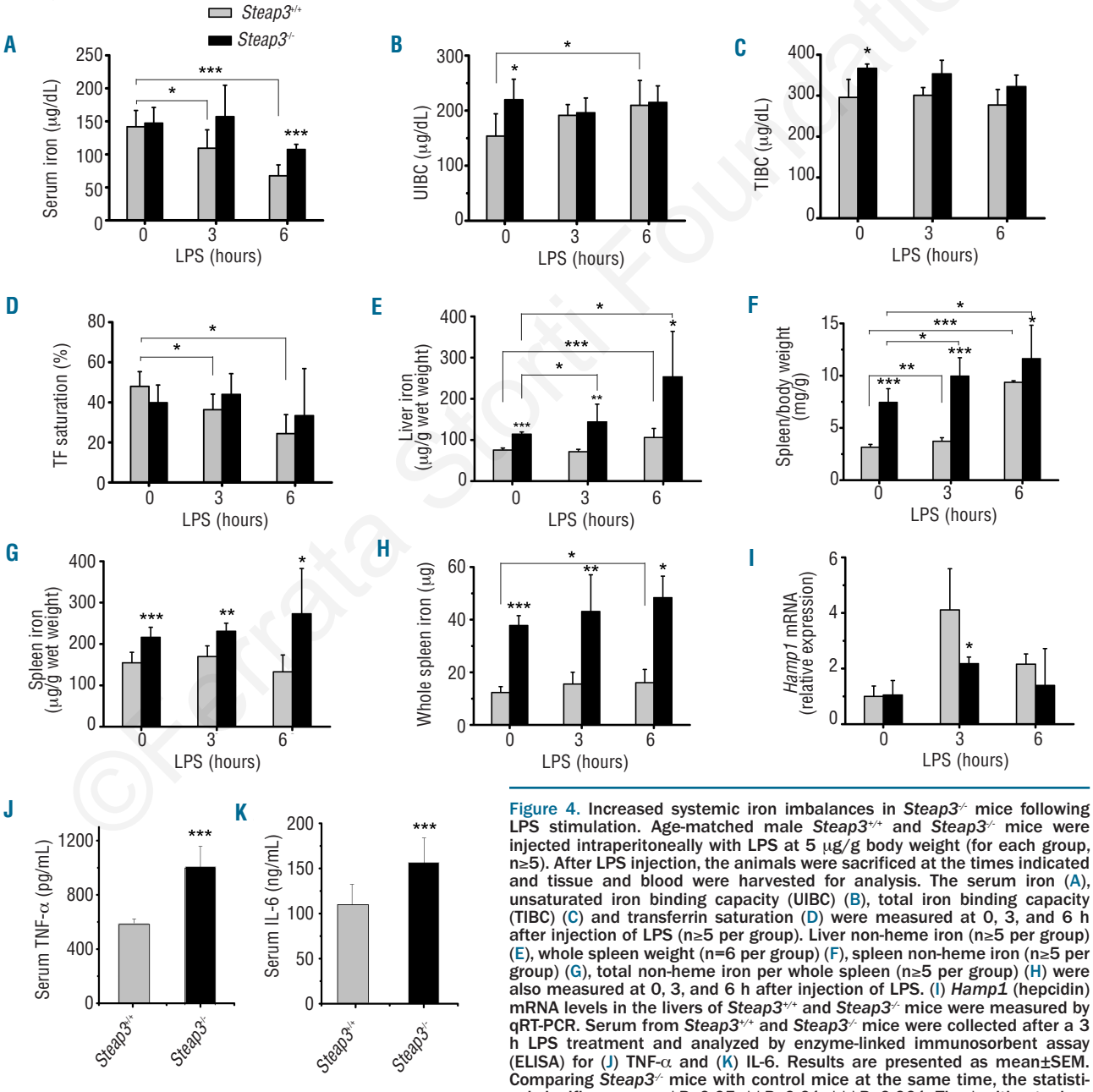

Figure 4. Increased systemic iron imbalances in *Steap3*$^{-/-}$ mice following LPS stimulation. Age-matched male *Steap3*$^{+/+}$ and *Steap3*$^{-/-}$ mice were injected intraperitoneally with LPS at 5 μg/g body weight (for each group, n≥5). After LPS injection, the animals were sacrificed at the times indicated and tissue and blood were harvested for analysis. The serum iron (A), unsaturated iron binding capacity (UIBC) (B), total iron binding capacity (TIBC) (C) and transferrin saturation (D) were measured at 0, 3, and 6 h after injection of LPS (n≥5 per group). Liver non-heme iron (n≥5 per group) (E), whole spleen weight (n=6 per group) (F), spleen non-heme iron (n≥5 per group) (G), total non-heme iron per whole spleen (n≥5 per group) (H) were also measured at 0, 3, and 6 h after injection of LPS. (I) *Hamp1* (hepcidin) mRNA levels in the livers of *Steap3*$^{+/+}$ and *Steap3*$^{-/-}$ mice were measured by qRT-PCR. Serum from *Steap3*$^{+/+}$ and *Steap3*$^{-/-}$ mice were collected after a 3 h LPS treatment and analyzed by enzyme-linked immunosorbent assay (ELISA) for (J) TNF-α and (K) IL-6. Results are presented as mean±SEM. Comparing *Steap3*$^{-/-}$ mice with control mice at the same time, the statistical significance was **P*<0.05, ***P*<0.01, ****P*<0.001. The * without a horizontal line represents the comparison with a wild-type sample at the same time point.

control and *Steap3* macrophages with regard to poly(I:C)-induced expression of *IFN-β* mRNA. This observation suggests that the functions of TRIF and signal transducing molecules distal to TRIF are unaffected by *Steap3* deficiency.

There are several aspects of the TLR4-specific activation of TRIF-dependent signals that could be affected by *Steap3* deficiency in macrophages, including abnormal cellular iron distribution, TLR4 endocytosis, TRAM endocytosis, TLR4/TRAM interactions, TRAM expression, and TRAM/TRIF interactions.[38] Steap3 might take part in these processes directly. Previous work had shown that *Hfe*, a gene that encodes the human hemochromatosis protein, whose deficiency leads to low cellular iron, might contribute to impair signaling through the TRAM/TRIF pathway.[39] In our study, although we observed the same iron content in *Steap3*$^{-/-}$ macrophages, the cytosolic available iron was much lower as the expression of ferritin was decreased in *Steap3*$^{-/-}$ macrophages before and after stimulation by either LPS or poly(I:C) (*Online Supplementary Figure S2A,B*). This may have led to impaired activation of the TRAM/TRIF pathway. The high iron concentrations could have enhanced TLR4-mediated, TRAM/TRIF-dependent pathways as we observed that the expression of IFN-β, MCP-5, IP-10 and phosphorylation of STAT1 were significantly enhanced after treatment with FAC during LPS stimulation.

In both *TRAM* knock-out mice and *TRIF* knock-out mice, impairment of the TRAM/TRIF-dependent pathway led to decreased production of TNF-α, IL-6, and IFN-β.[25,27] However, TNF-α and IL-6, which could also be induced by a TRAM/TRIF pathway other than the Myd88-dependent one, were not decreased in *Steap3*$^{-/-}$ macrophages after LPS stimulation. We suspect that the low cytosolic iron in *Steap3*$^{-/-}$ macrophages might contribute to the impaired production of IFN-β. Further investigation is required to

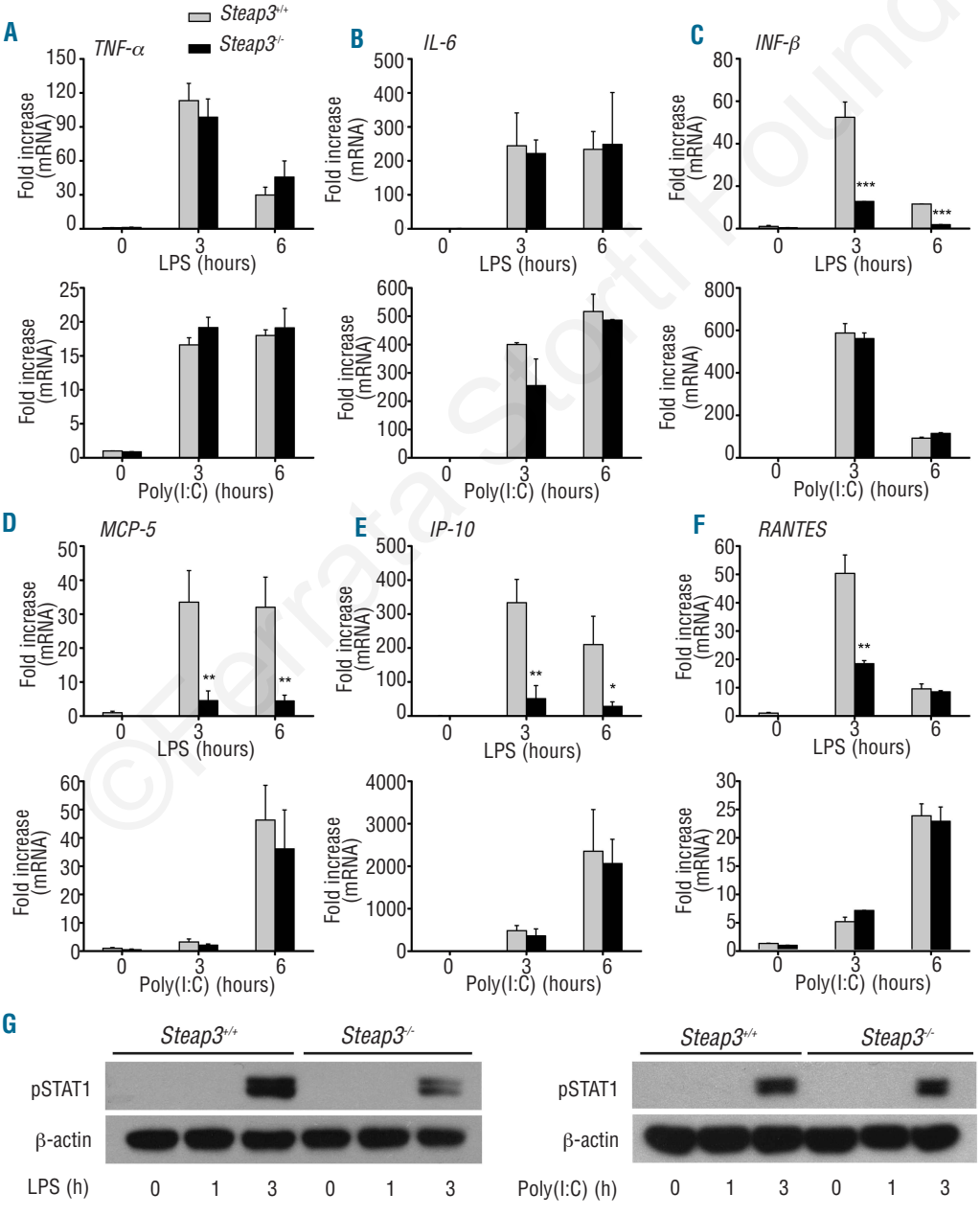

Figure 5. Impairment of TLR4-mediated inflammatory responses in *Steap3*$^{-/-}$ macrophages. BMDMs of control and *Steap3*$^{-/-}$ mice were treated with LPS (100 ng/mL) or poly(I:C) (5 μg/mL) for the indicated time. The mRNA expression levels of *TNF-α* (A), *IL-6* (B), *IFN-β* (C), *MCP-5* (D), *IP-10* (E) and *RANTES* (F) were measured by qRT-PCR at 0, 3, 6 h after LPS or poly(I:C) stimulation. (G) Phosphorylation of STAT1 was examined by western blotting at 0, 1, and 3 h after LPS or Poly(I:C) stimulation. Data represent mean ± SEM. Similar results were obtained in three separate experiments. **P*<0.05, ***P*<0.01, ****P*<0.001.

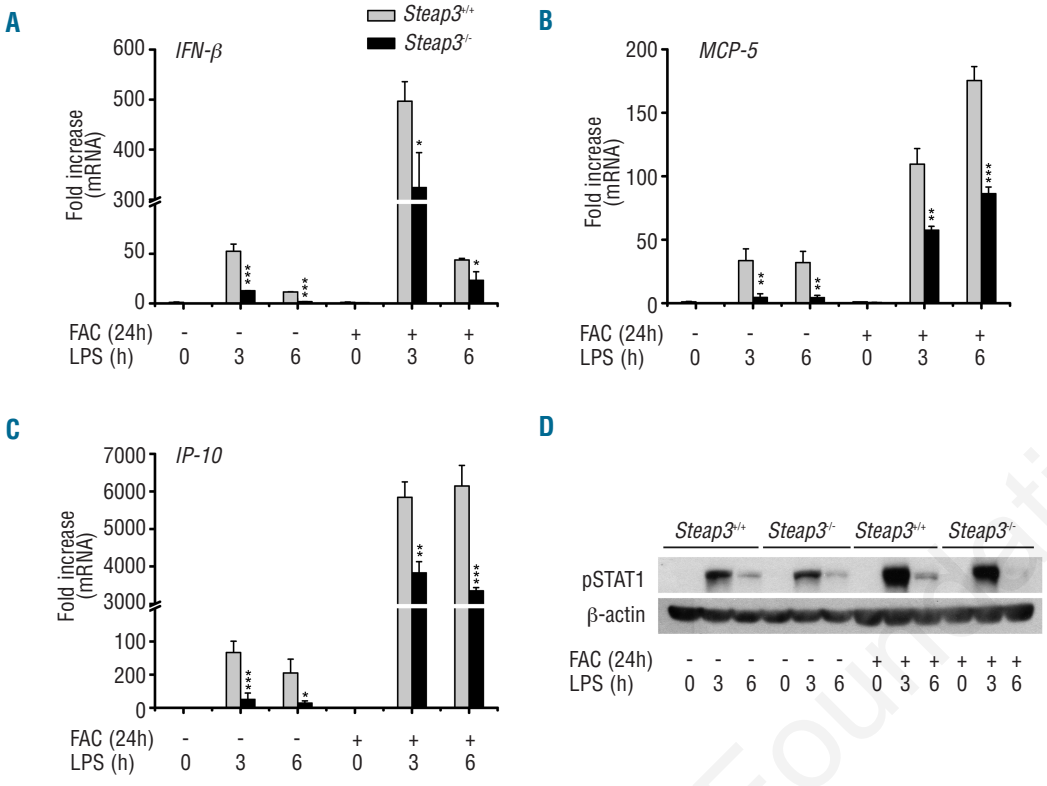

Figure 6. Deletion of Steap3 inhibits iron-enhanced, TLR4-mediated inflammatory responses. BMDMs of control and *Steap3*$^{-/-}$ mice were stimulated with LPS (100 ng/mL) before or after FAC (100 mM) treatment for 24 h. The mRNA expression levels of *IFN-β* (A), *MCP-5* (B), and *IP-10* (C) were measured by qRT-PCR at 0, 3, and 6 h after LPS stimulation. (D) Phosphorylation of STAT1 was examined by western blot at 0, 3, and 6 h after LPS stimulation. Data represent mean ± SEM. Similar results were obtained in three separate experiments. **P*<0.05, ***P*<0.01, ****P*<0.001.

determine whether Steap3 is involved in the TLR4-mediated, Myd88-dependent signaling pathway. The most likely mechanism by which Steap3 might regulate TLR4 signaling is through altered endocytosis of TLR4[40] in the absence of Steap3, resulting in more TLR4 in the cell membrane after LPS stimulation. Thus, the impaired TRAM/TRIF-dependent pathway might be accompanied by competitive enhancement of the Myd88-dependent pathway. These proposed mechanisms will need to be confirmed by further investigations. Future studies will be required to determine whether Steap3 itself or other STEAP family members are directly involved in these inflammatory responses.

It seemed paradoxical that inactivation of *Steap3* led to impaired TLR4-mediated inflammatory responses when *Steap3* could be down-regulated by LPS. It is possible that due to negative feedback *Steap3* is down-regulated by LPS in order to decrease the inflammatory response. Furthermore, we used different stimuli including poly(I:C) (a TLR3 agonist) and pam3CSK4 (a TLR2 agonist), and found that in both cases Steap3 could be down-regulated. However, *IFN-β* expression was the same in wild-type and *Steap3*$^{-/-}$ macrophages stimulated with poly(I:C). Thus, the down-regulation of *Steap3* by stimulation seemed to be independent of the impaired inflammatory responses caused by decreased cytosolic iron. We propose that this might be part of a protective mechanism resembling the way that Fpn1 allows organisms to keep iron in the cell in order to avoid it being used by pathogens such as bacteria or viruses. Though the down-regulation of *Steap3* might not be related to the TLR4-mediated pathway, we could not exclude the possibility that *Steap3* takes part in the TLR4 signaling pathway.

In conclusion, our findings revealed important roles for Steap3 in the coordinate regulation of iron homeostasis and inflammatory responses, which helps to shed light on interactions between iron homeostasis and innate immunity.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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