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A role for hepcidin in the anemia caused by *Trypanosoma brucei* infection

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ABSTRACT

Trypanosomiasis is a parasitic disease affecting both humans and animals in the form of Human African Trypanosomiasis and Nagana disease, respectively. Anemia is one of the most common symptoms of trypanosomiasis, and if left unchecked can cause severe complications and even death. Several factors have been associated with the development of this anemia, including dysregulation of iron homeostasis, but little is known about the molecular mechanisms involved. Here, using murine models, we study the involvement of hepcidin, the key regulator of iron metabolism and an important player in the development of anemia of inflammation. Our data show two stages for the progression of anemia, to which hepcidin contributes a first stage when anemia develops, with a likely cytokine-mediated stimulation of hepcidin and subsequent limitation in iron availability and erythropoiesis, and a second stage of recovery, where the increase in hepcidin then declines due to the reduced inflammatory signal and increased production of erythroid regulators by the kidney, spleen and bone marrow, thus leading to an increase in iron release and availability, and enhanced erythropoiesis. In agreement with this, in hepcidin knockout mice, anemia is much milder and its recovery is complete, in contrast to wild-type animals which have not fully recovered from anemia after 21 days. Besides all other factors known to be involved in the development of anemia during trypanosomiasis, hepcidin clearly makes an important contribution to both its development and recovery.

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Introduction

African trypanosomes are extracellular protozoan parasites transmitted by the hematophagous tsetse fly (*Glossina spp*), and are responsible for debilitating medical and veterinary diseases in sub-Saharan Africa.[1] *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense* infect humans and are responsible for the fatal Human African Trypanosomiasis, also known as sleeping sickness.[2] *Trypanosoma brucei brucei* is responsible for animal trypanosomiasis, which mostly affects cattle.[2]

One of the most common complications of trypanosomiasis is anemia, both in humans and animals,[3-5] which in conjunction with other symptoms can be a major cause of death if left untreated, particularly in livestock.[6] Over the years, several causes for this anemia have been described, and these include both parasite and host-associated factors.[7-9] Erythrophagocytosis by activated liver and spleen myeloid cells has been identified as a major contributor to erythrocyte clearance. In addition, the lipid composition of erythrocytes is altered during trypanosome infection and these are preferentially phagocytosed.[8]

Among the parasite factors that contribute to anemia are the expression of extra-

cellular products, such as hemolysins,[10,11] direct mechanical erythrocyte injury,[12] lipid peroxidation,[13-15] and extracellular vesicles that can fuse with erythrocytes resulting in rapid clearance and anemia.[16] Furthermore, although there are clear indications that iron metabolism has a significant role in the establishment of anemia during trypanosomal infections,[17,18] the overall molecular mechanisms that lead to it are still poorly understood, and this includes, in particular, the involvement of hepcidin.

Hepcidin is a small antimicrobial peptide and a key regulator of iron metabolism.[19-21] During infectious/inflammatory processes, hepcidin leads to a systemic decrease in iron mobilization by blocking iron release from hepatocytes, enterocytes and macrophages. This impacts the proliferation of the pathogens but also affects the host by impairing erythropoiesis. The scarcity of iron and the subsequent impairment of erythropoiesis are thought to lead to a condition known as anemia of inflammation. This mechanism of response has been established for several bacterial infections[22-26] and some intracellular parasites.[27,28] However, studies of hepcidin involvement in the development of anemia in infections with extracellular parasites are extremely limited.

The present study was undertaken to determine the possible role of hepcidin in the regulation of iron metabolism during trypanosomal infections and its contribution to the onset, development and recovery from anemia.

Methods

Mice, parasites and infections

Five-week-old C57BL/6 and BALB/c female mice were purchased from Charles River Laboratories (Saint-Germain-Nuelles, France). Female hepcidin knockout (*Hamp*$^{-/-}$) mice[29] were bred at the institute facilities. The *Trypanosoma brucei brucei*, GVR35 strain, was used to infect the mice. All experiments were carried out in accordance with the IBMC.INEB Animal Ethics Committees and the Portuguese National Authorities for Animal Health guidelines according to the statements on the directive 2010/63/EU of the European Parliament and Council.

Hematologic and serum parameters, tissue iron content

Hematologic and serum parameters were blindly determined by a certified laboratory (CoreLab, Centro Hospitalar do Porto, Portugal). Liver and spleen iron content was evaluated by the bathophenanthroline method[30] and Perls staining.

Cytokine profile analysis

Cytokine levels were measured in the serum using the BD CBA Mouse Inflammation Kit (BD Biosciences, San Jose, CA, USA).

Flow cytometry

Bone marrow (BM) cells were stained with anti-CD3e (17A2), anti-CD19 (6D5), anti-TER119, anti-CD71 (RI7217), anti-CD11c (N418), and anti-CD11b (M1/70) antibodies, and run in a BD FACSCanto II Flow Cytometer (BD Biosciences). Data were analyzed with FlowJo software (FlowJo LCC, Ashland, OR, USA).

RNA isolation and cDNA synthesis

Total RNA was isolated from tissues and cells with the PureLink RNA Mini Kit (Thermo Fisher Scientific) and converted to cDNA using the NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal).

Analysis of gene expression by quantitative-polymerase chain reaction

Relative levels of several genes mRNAs were quantified in relevant organs of control and infected animals, by quantitative-polymerase chain reaction (qPCR). The comparative CT method ($2^{-\Delta\Delta CT}$ method) was used to analyze gene expression levels.

Analysis of ferroportin levels by Western blot

Levels of FPN1 protein were evaluated in the liver, spleen and duodenum of C57BL/6 and *Hamp*$^{-/-}$ mice by Western blot, with GAPDH being used as housekeeping protein. Primary antibodies used were rabbit anti-Ferroportin/SLC40A1 (Novus, Littletown, CO, USA; catalog #NPB1-21502), (1:1000) 1 hour (h) RT, rabbit anti-GAPDH (Abcam, Cambridge, UK, catalog #EPR16891), (1:1000) 1 h RT.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, USA). Multiple comparisons were performed with one-way ANOVA and *post hoc* Student Newman-Keuls test. $P<0.05$ was considered statistically significant.

Further details of the study methods are available in the *Online Supplementary Appendix*.

Results

T.b. brucei infection in mice leads to macrocytic anemia, decreased erythropoietic activity and iron redistribution

The course of the infection with *T.b. brucei* GVR35 strain expressing luciferase was followed in BALB/c mice by bioluminescence imaging and counting the parasites in the blood (*Online Supplementary Figure S1*). A significant reduction in the number of red blood cells (RBC), reticulocytes, hematocrit and hemoglobin levels was observed up to day 7, with a gradual return to normal levels (Figure 1A-D), indicating an early onset of acute anemia, followed by a later recovery. A significant increase in the mean corpuscular volume (MCV) was also observed, indicative of macrocytic anemia (Figure 1E). Alterations in the development of erythroid lineage in the BM were evaluated by flow cytometry. Overall, a decreased total number of mature and developing erythrocytes was observed (Figure 1F), as well as decreased numbers of pro-erythroblasts (Figure 1G), basophilic erythroblasts (Figure 1H), and polychromatic erythroblasts (Figure 1, panel I).

Trypanosomal infection also caused a significant decrease in circulating serum iron levels (Figure 1J), transferrin saturation (Figure 1K), and increased total iron binding capacity (TIBC) (Figure 1L) and serum ferritin (Figure 1M), highlighting the inflammatory status of the animals and thus, indirectly, a condition of anemia of inflammation. The lower systemic iron concentration was accompanied by the accumulation of iron in the liver (Figure 1N) and spleen (Figure 1, panel O).

Circulating cytokine levels indicate the development of an acute infection

We evaluated the impact of *T.b. brucei* infection in the expression of several inflammatory cytokines. IL-6 levels were elevated as early as 1 day post infection and remained high up to day 7, with a gradual recovery to control levels (Figure 2A). IL-6 not only acts as a pro-inflam-

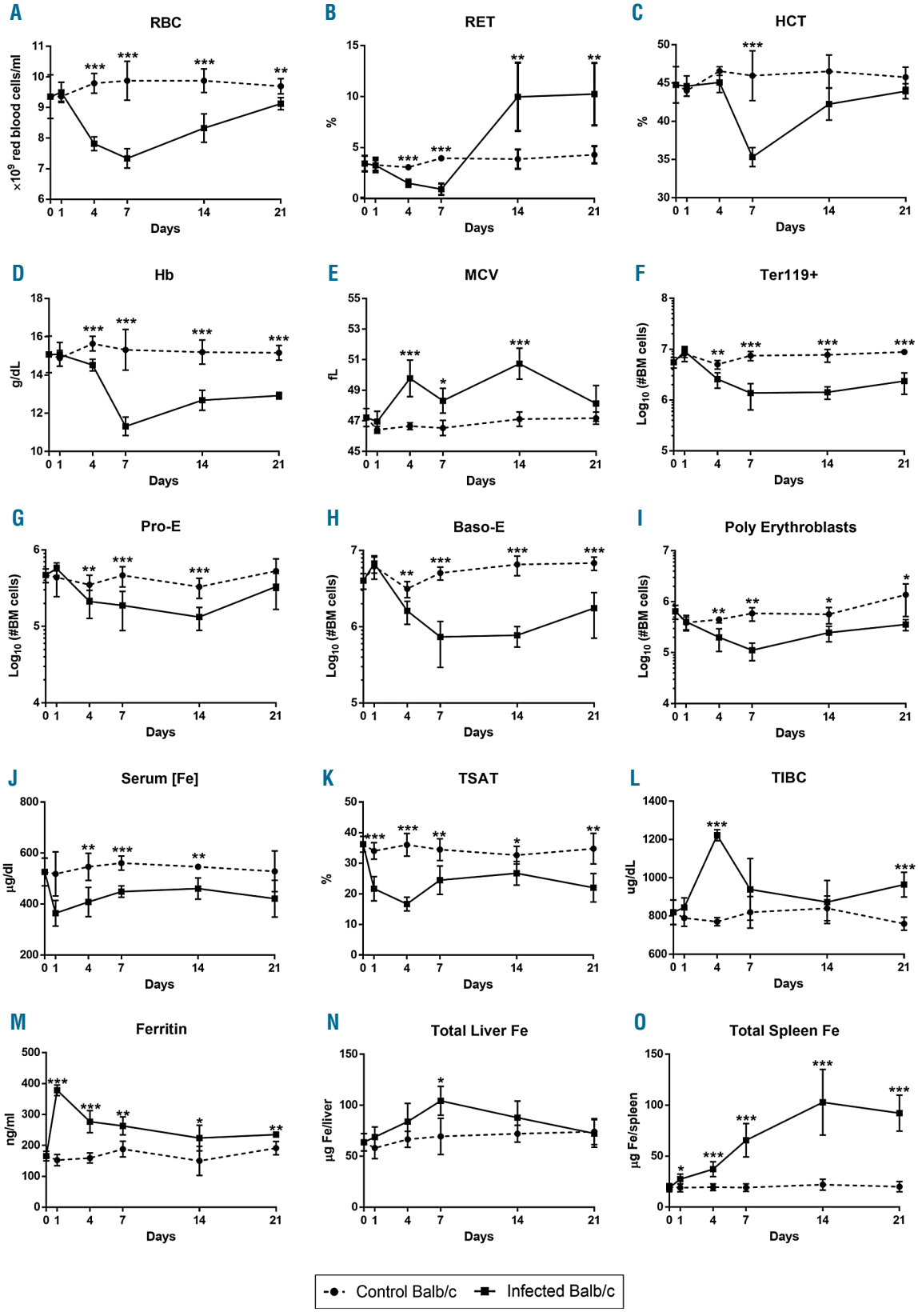

Figure 1. Hematologic parameters, bone marrow (BM) erythroid populations, serum parameters and tissue iron content in BALB/c mice. Blood, serum and tissue samples were collected at 1, 4, 7, 14 and 21 days post infection with *T.b. brucei*. Hematologic parameters were blindly determined by a certified laboratory and BM cells were isolated, stained with antibodies for erythroid populations and analyzed by flow cytometry. Serum parameters were blindly determined by a certified laboratory and iron content in the liver and spleen was measured by the bathophenanthroline method. (A) Red blood cells (RBC), (B) reticulocytes (RET), (C) hematocrit (HCT), (D) hemoglobin (Hb), (E) mean corpuscular volume (MCV), (F) total erythroid cells (Ter119+), (G) pro-erythroblasts (Pro-E), (H) basoerythroblasts (Baso-E), (I) polychromatic erythroblasts (Poly-E), (J) serum iron [Fe], (K) transferrin saturation (TSAT), (L) total iron binding capacity (TIBC), (M) serum ferritin, (N) total liver iron [Fe], (O) total spleen iron [Fe]. Values are represented as mean±standard deviation (n=5). **P*<0.05, ***P*<0.01, and ****P*<0.001 were considered statistically significant.

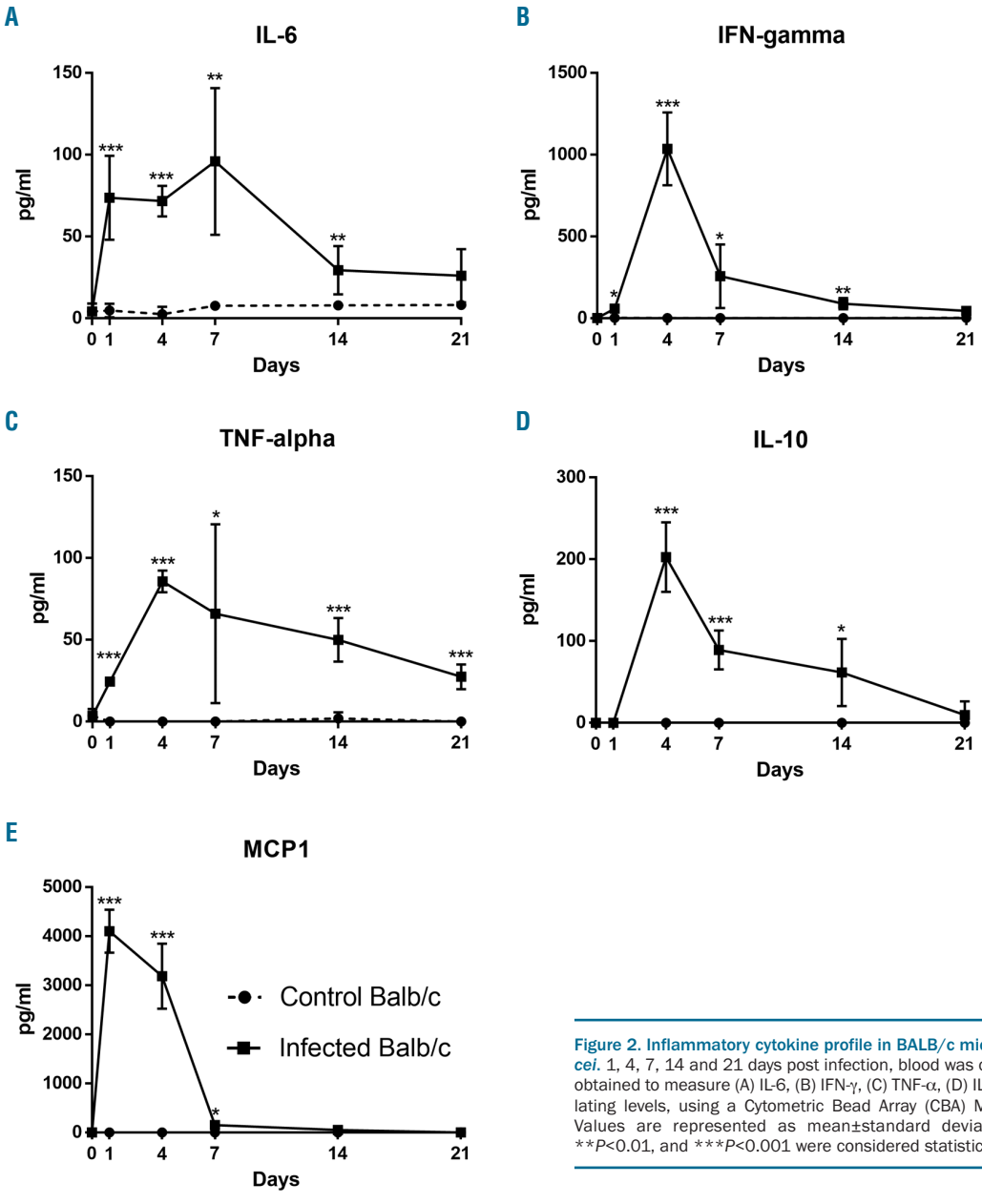

Figure 2. Inflammatory cytokine profile in BALB/c mice infected with *T.b. brucei*. 1, 4, 7, 14 and 21 days post infection, blood was collected and serum was obtained to measure (A) IL-6, (B) IFN-γ, (C) TNF-α, (D) IL-10, and (E) MCP1 circulating levels, using a Cytometric Bead Array (CBA) Mouse Inflammation Kit. Values are represented as mean±standard deviation (n=5). **P*<0.05, ***P*<0.01, and ****P*<0.001 were considered statistically significant.

matory cytokine, inducing the expression of other cytokines and several acute phase proteins, but is also an important hepcidin inducer, thus potentially impacting iron metabolism during infection. Circulating levels of IFN-γ, TNF-α and IL-10 (Figure 2B-D) followed a similar pattern, with an increase up to day 4 followed by a decrease throughout the course of the experiment, with both IFN-γ and IL-10 returning to normal levels at 21 days post infection. Additionally, high levels of MCP-1 were observed as early as day 1 post infection, remaining high at day 4, and rapidly decreasing to almost normal levels (Figure 2E).

Evaluation of gene expression indicates an early onset of acute anemia and later recovery

Expression of hepcidin and other iron-related and hematopoietic genes was evaluated in the liver, spleen, kidney and BM. In the liver, a gradual increase in *Hamp1* expression was observed up to day 7 post infection, followed by a decrease, reaching lower than control levels at day 21 (Figure 3A). Ferroportin (*Fpn1*) expression was significantly down-regulated as early as day 1, with a gradual recovery to control levels followed by an incremental upregulation up to day 21 post infection (Figure 3B). Transferrin (*Tf*) (Figure 3C) and ferritin H (*Fth1*) (Figure 3D) were also significantly up-regulated at the early stages of infection, returning to control levels before day 14, coinciding with the increases in TIBC and circulating ferritin levels, respectively (Figure 1L and M).

In the spleen, *Hamp1* was observed to be up-regulated in the earlier days of infection, returning to control levels at day 7 and with a slight decrease at day 21 (Figure 3E). Expression levels of *Fth1* started to increase at day 4, peaked at day 7, and then gradually decreased towards the end of the experiment (Figure 3G). Similar patterns of upregulation were observed for *Fpn1, Hbb, Epor, Erfe* and *Twsg1* (Figure 3F, H-K), with significant increases in expression starting as early as day 4, and maintained up to day 21. In the kidney, an increase in *Hamp1* expression

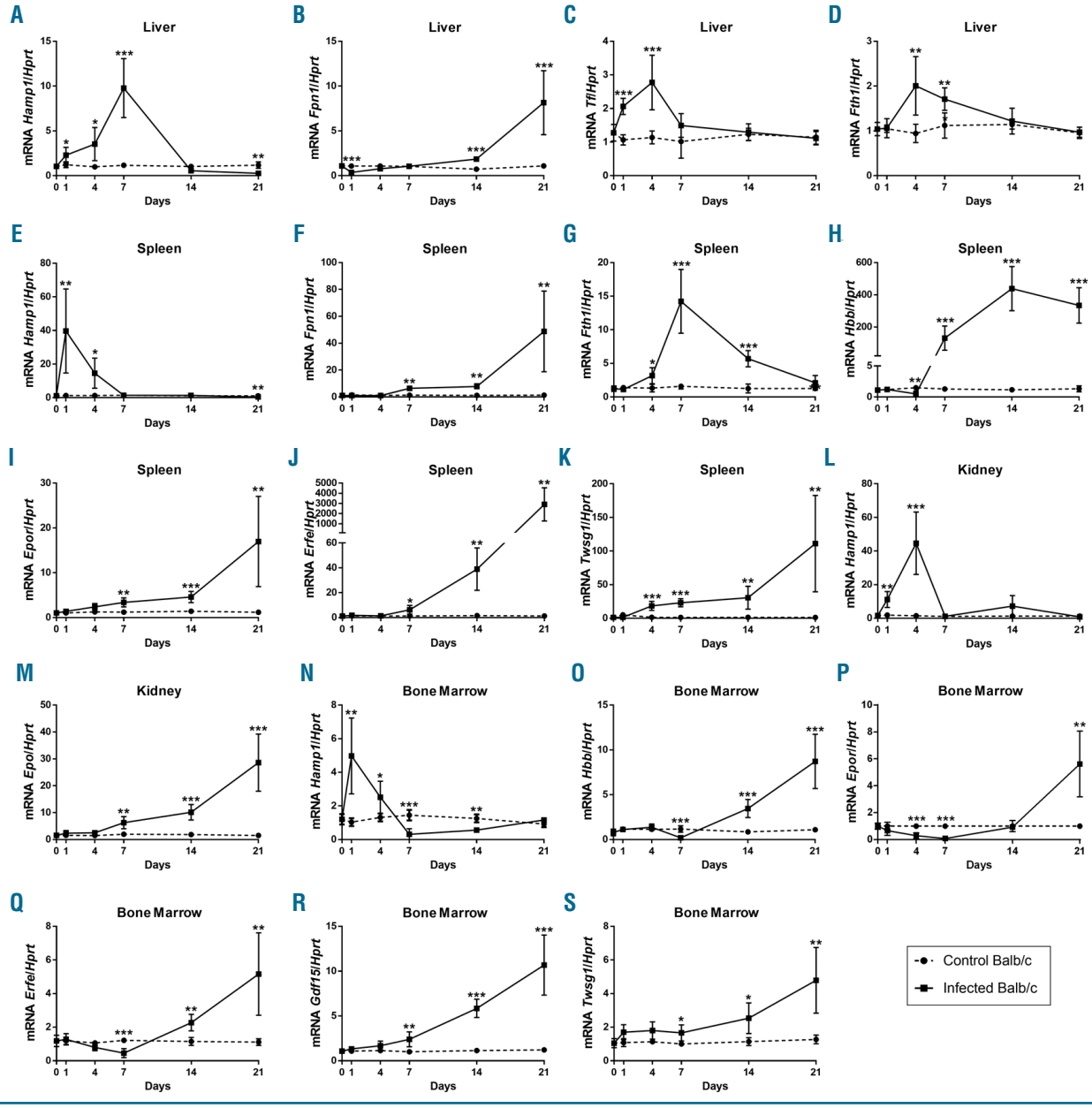

Figure 3. Gene expression in the liver, spleen, kidney and bone marrow after experimental infection of BALB/c mice with *T.b. brucei*. Relative mRNA expression of several genes was measured 1, 4, 7, 14, and 21 days post infection, by real-time polymerase chain reaction. Liver (A) *Hamp1*, (B) *Fpn1*, (C) *Tf*, (D) *Fth1;* spleen (E) *Hamp1*, (F) *Fpn1*, (G) *Fth1*, (H) *Hbb*, (I) *Epor*, (J) *Erfe*, (K) *Twsg1*; kidney (L) *Hamp1*, (M) *Epo*; bone marrow (N) *Hamp1*, (O) *Hbb*, (P) *Epor*, (Q) *Erfe*, (R) *Gdf15*, (S) *Twsg1*. Values are represented as mean±standard deviation (n=5). Differences from the control groups were considered significant at **P*<0.05, ***P*<0.01, and ****P*<0.001.

was observed starting day 1, peaking at day 4, and returning to control levels at day 7 (Figure 3L). Levels of *Epo* were up-regulated throughout the duration of the experiment (Figure 3M).

Finally, in the BM, *Hamp1* expression was found to be increased as early as day 1, followed by a gradual down-regulation up to day 7, and a recovery to normal values towards day 21 (Figure 3N). For *Hbb, Epor* and *Erfe*, a similar pattern of expression was observed, with decreased expression at day 7, followed by gradual increases, and reaching maximum levels at day 21 (Figure 3, panels O-Q). *Gdf15* and *Twsg1* expressions gradually increased throughout the course of the infection, reaching maximum levels at day 21 (Figure 3R and S).

Hepcidin contributes to the development of anemia in trypanosomal infections

Gene expression profiles indicate that hepcidin might be involved in the development of anemia during trypanosomal infection. In order to investigate this, we performed experimental infections in hepcidin deficient (*Hamp*$^{-/-}$) mice. No significant differences were found in the parasitemias or total parasite burdens between *Hamp*$^{-/-}$ and C57BL/6 mice (*Online Supplementary Figure S2*). Hematologic parameters show development of anemia, with a steady decline of RBC number, hematocrit and hemoglobin levels up to day 7 post infection, followed by a gradual recovery (Figure 4A-D). However, whereas in C57BL/6 mice parameters never fully recover to normal values, in *Hamp*$^{-/-}$ mice there is a

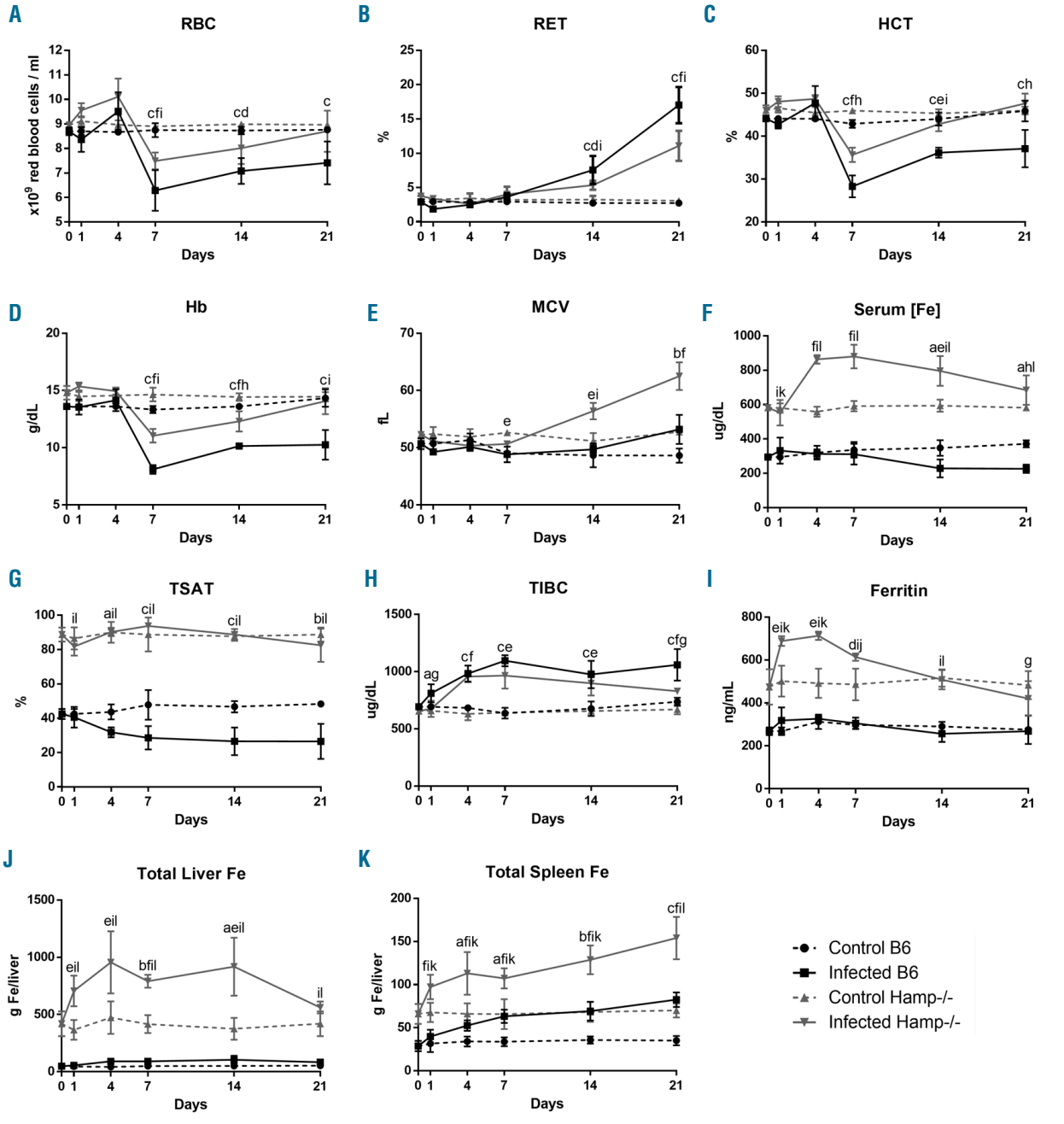

Figure 4. Hematologic and serum parameters and tissue iron content in C57BL/6 and *Hamp*$^{-/-}$ mice. Blood, serum and tissue samples were collected at 1, 4, 7, 14 and 21 days post-infection with *T.b. brucei*. Hematologic and serum parameters were blindly determined by a certified laboratory and tissue iron content was measured by the bathophenanthroline method. (A) Red blood cells (RBC), (B) reticulocytes, (C) hematocrit (HCT), (D) hemoglobin (Hb), (E) mean corpuscular volume (MCV), serum iron [Fe], (G) transferrin saturation (TSAT), (H) total iron binding capacity (TIBC), (I) serum ferritin, (J) total liver iron [Fe], (K) total spleen iron [Fe]. Values are represented as mean±standard deviation (n=5). Differences among groups were considered significant at $P<0.05$, $P<0.01$, and $P<0.001$, represented respectively by the letters a, b, c between control and infected C57BL/6 mice, d, e, f between control and infected *Hamp*$^{-/-}$ mice, g, h, i between infected groups and j, k, l between control groups.

complete reversal of anemia and a return to normal conditions, indicating a more severe anemia was established in the wild-type animals (Figure 4A, C and D). A significant increase in reticulocyte numbers and MCV was also observed at later stages of infection in both wild-type and knockout mice (Figure 4B and E). Variations in serological parameters and tissue iron content in C57BL/6 mice were mostly comparable to BALB/c, with decreases in serum iron levels and transferrin saturation (Figure 4F and G), increases in TIBC (Figure 4H), as well as in liver and spleen iron (Figure 4J and K, and *Online Supplementary Figure S3*) with no changes in ferritin levels (Figure 4, panel I). In *Hamp*$^{-/-}$ mice, only a similar increase in TIBC (Figure 4H) was observed, as well as increases in liver and spleen iron (Figure 4J and K) with no changes in transferrin saturation (Figure 4G). Furthermore, despite the higher levels of serum iron and circulating ferritin, when compared with C57BL/6, increases in both parameters were also observed in the early days of infection, with recoveries to near normal towards the end (Figure 4F and I).

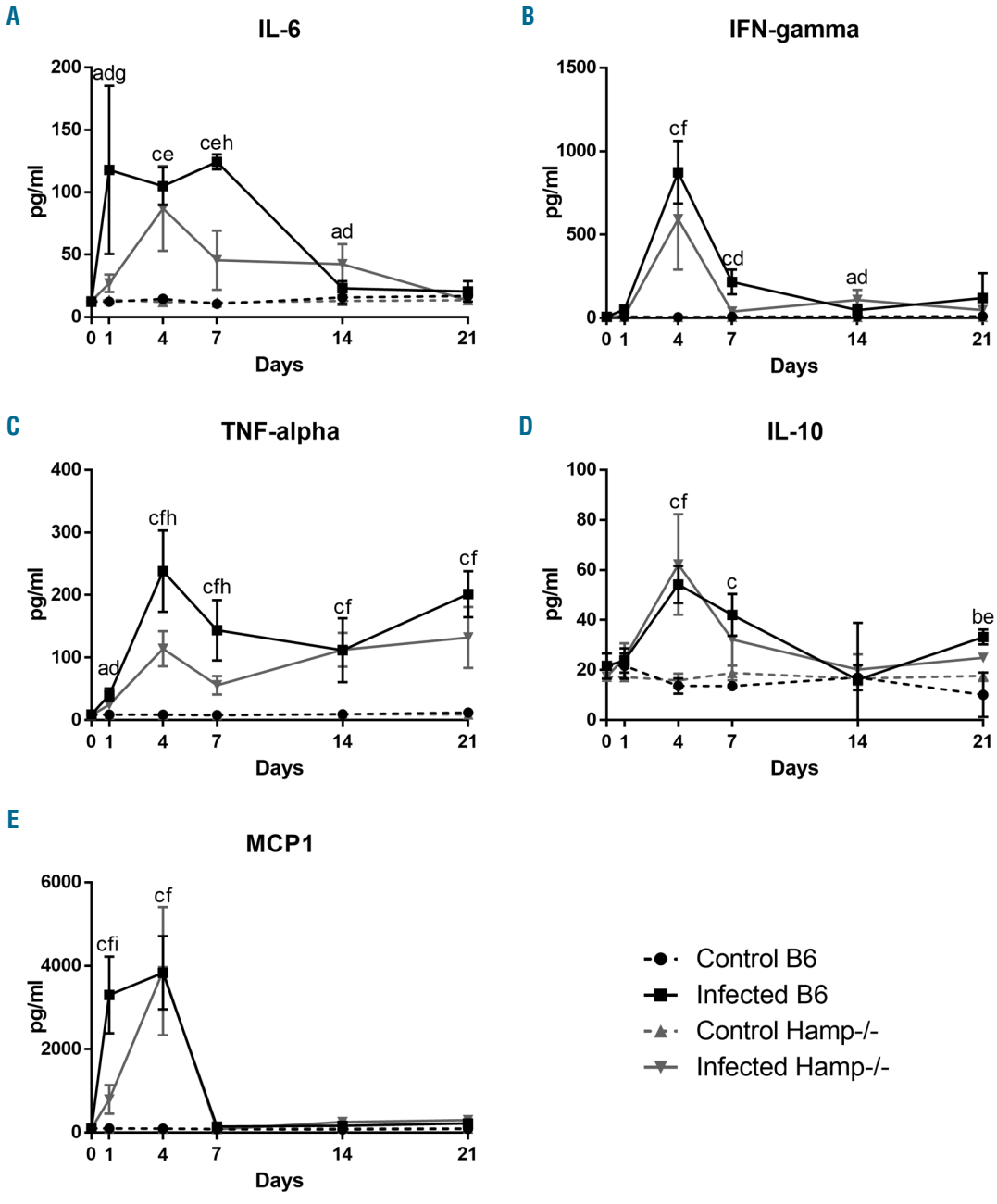

Figure 5. Inflammatory cytokine profile in C57BL/6 and *Hamp*$^{-/-}$ mice infected with *T.b. brucei*. 1, 4, 7, 14 and 21 days post infection, blood was collected and serum was obtained to measure (A) IL-6, (B) IFN-γ, (C) TNF-α, (D) IL-10, and (E) MCP1 circulating levels, using a Cytometric Bead Array (CBA) Mouse Inflammation Kit. Values are represented as mean±standard deviation (n=5). Differences among groups were considered significant at $P<0.05$, $P<0.01$, and $P<0.001$, represented respectively by the letters a, b, c between control and infected C57BL/6 mice, d, e, f between control and infected *Hamp*$^{-/-}$ mice, g, h, i between infected groups and j, k, l between control groups.

Circulating cytokine levels in both C57BL/6 and *Hamp*$^{-/-}$ mice were mostly comparable to BALB/c. IL-6 levels increased as early as day 1 post infection and remained high up to day 7 or day 14 (in C57BL/6 and *Hamp*$^{-/-}$) with a subsequent gradual recovery to control levels (Figure 5A). Circulating levels of IFN-γ, TNF-α and IL-10 (Figure 5B-D) also followed similar patterns, with an increase up to day 4 followed by a decrease. Additionally, high levels of MCP1 were observed as early as day 1 post infection, reaching maximum levels at day 4 and rapidly decreasing to normal levels at day 7 post infection (Figure 5E).

Gene expression was also evaluated in the liver, spleen and kidney. Hepcidin and ferroportin liver expression profiles in C57BL/6 mice were similar to what was observed for BALB/c, with an increase of hepcidin up to day 7 followed by a decrease to lower than normal levels, and with ferroportin mirroring hepcidin (Figure 6A and B), coinciding with the early onset of anemia and the subsequent recovery. No discernible hepcidin expression was observed in the liver of *Hamp*$^{-/-}$ animals, and the increase in ferroportin expression was maintained throughout the duration of the experiment (Figure 6A and B). In the spleen, an increase in *Hbb* (Figure 6C) and *Twsg1* (Figure 6E) was observed in both wild-type and

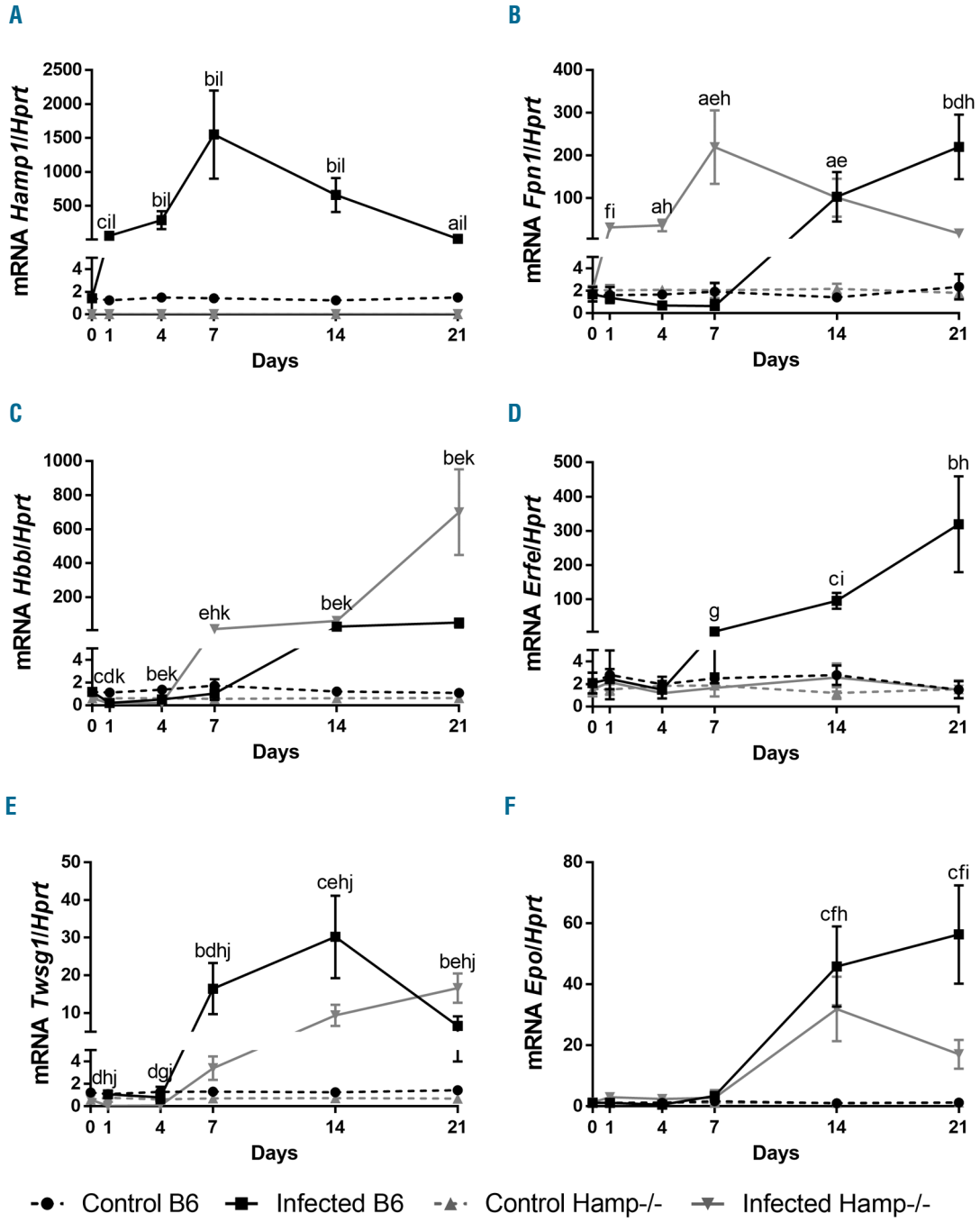

Figure 6. Gene expression in the liver, spleen and kidney of C57BL/6 and *Hamp*$^{-/-}$ mice after experimental infection with *T.b. brucei*. Relative mRNA expression of several genes was measured 1, 4, 7, 14, and 21 days post infection, by real-time poymerase chain reaction. (A) liver *Hamp1*, (B) liver *FPn1*, (C) spleen *Hbb*, (D) spleen *Erfe*, (E) spleen *Twsg1*, (F) kidney *Epo*. Values are represented as means ± standard deviation (n=5). Differences among groups were considered significant at $P<0.05$, $P<0.01$, and $P<0.001$, represented respectively by the letters a, b, c between control and infected C57BL/6 mice, d, e, f between control and infected *Hamp*$^{-/-}$ mice, g, h, i between infected groups and j, k, l between control groups.

knockout mice. Increases in the expression of *Epo* were also observed in the kidney of both C57BL/6 and *Hamp*$^{-/-}$ infected mice, remaining elevated throughout the infection in C57BL/6 mice, but decreasing at day 21 in *Hamp*$^{-/-}$ mice (Figure 6F). Despite the drive for enhanced erythropoiesis being present in both wild-type and knockout mice, *Erfe* was only up-regulated in C57BL/6 mice, similar to BALB/c, but no changes in expression were observed in *Hamp*$^{-/-}$ mice (Figure 6D). Ferroportin protein levels were also measured in the liver, spleen and duodenum of C57BL/6 and *Hamp*$^{-/-}$. In the liver, variations in protein levels closely matched the variations in mRNA expression, with significant decreases in both C57BL/6 and *Hamp*$^{-/-}$ mice at days 1 and 4 followed by gradual recoveries towards day 21, although earlier and stronger in *Hamp*$^{-/-}$ (Figure 7A). In the spleen, ferroportin levels gradually decreased until they were no longer detected up to day 4, followed by a recovery up to day 21, but

starting earlier and reaching higher levels in *Hamp*$^{-/-}$, where ferroportin could already be observed at day 7, with much higher levels at day 21 (Figure 7B). No significant changes in ferroportin protein levels were observed in the duodenum (Figure 7C).

Discussion

Using a mouse model of trypanosomiasis (which has been shown to replicate the two stages of the disease[31] occurring in humans) we performed an integrated analysis

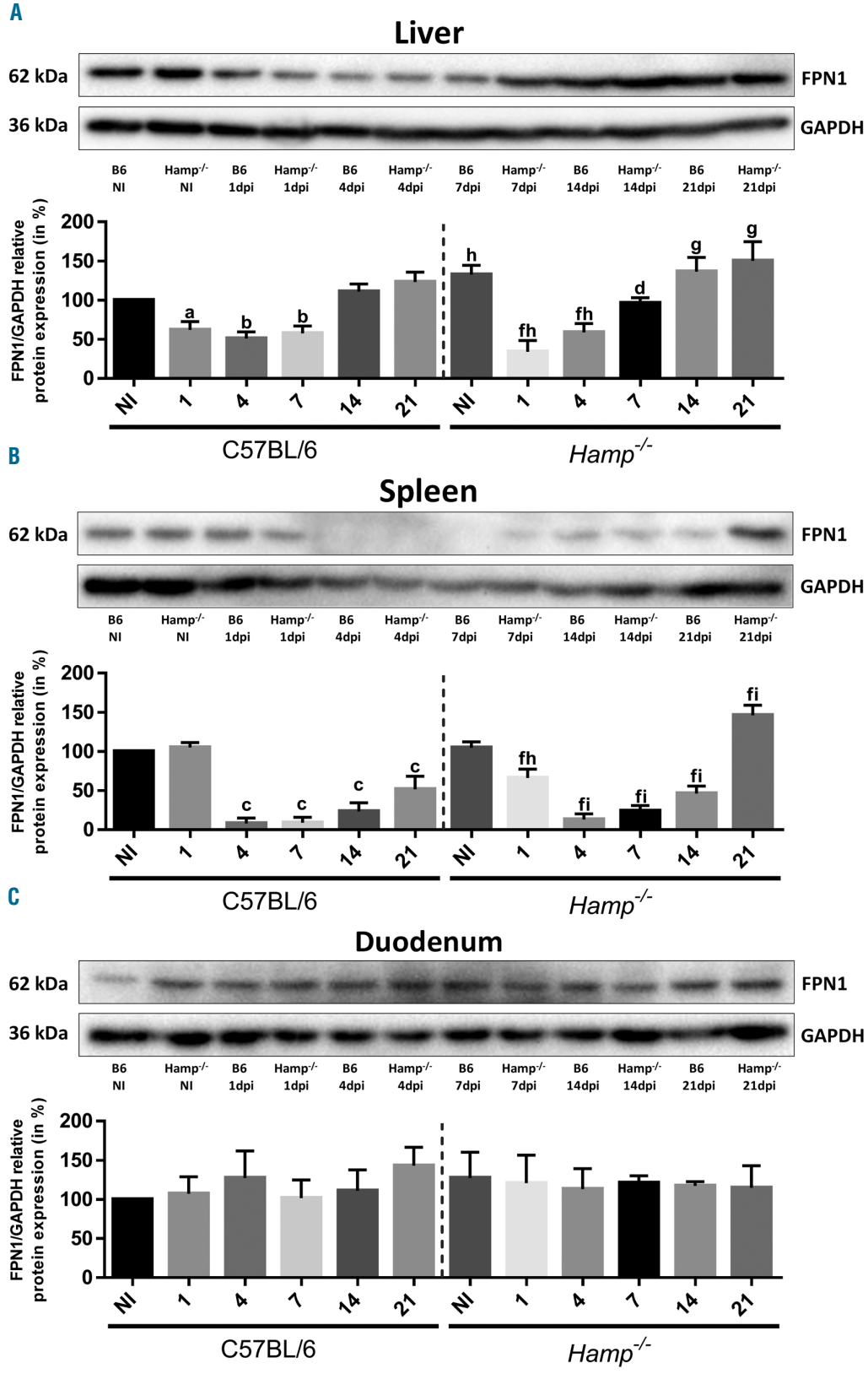

Figure 7. Ferroportin protein levels in the liver, spleen and duodenum of C57BL/6 and *Hamp*$^{-/-}$ mice during infection *T.b. brucei*, analyzed by Western blot. (A) Liver. (B) Spleen. (C) Duodenum. GAPDH was used as housekeeping protein. Graphs represent the densitometry analysis of FPN1 protein in each day post infection, normalized to GAPDH densitometry, expressed as percentage to non-infected C57BL/6 animals. Values are represented as mean±standard deviation (n=4). Differences among groups were considered significant at $P<0.05$, $P<0.01$, and $P<0.001$, represented respectively by the letters a, b, c between control and infected C57BL/6 mice, d, e, f between control and infected *Hamp*$^{-/-}$ mice and g, h, i between C57BL/6 control and *Hamp*$^{-/-}$ mice. NI: non-infected; dpi: days post infection.

on the establishment/progression of anemia and the molecular mechanisms involved in iron homeostasis. Furthermore, this study also evaluates the contribution of hepcidin (the key regulator of iron homeostasis[19,32]) to the anemia established during *T.b. brucei* infection. Different degrees of anemia severity in trypanosomal infections have been reported and these have been described as being dependent on the host and sub-species causing the infection.[3,7] In this study, an early decrease in several hematologic parameters such as the number of RBC and reticulocytes, hematocrit and hemoglobin levels, and an increase in MCV were seen in wild-type (BALB/c and C57BL/6) and in *Hamp*$^{-/-}$ (gene deficient in C57BL/6 background) mice. This was followed later by an increase in most parameters, never reaching normal levels in wild-type (BALB/c and C57BL/6) animals but fully recovering in *Hamp*$^{-/-}$ mice.

An in-depth investigation of several erythrocyte populations in the BM of BALB/c mice shows similar patterns in all of them, from the early immature pro-erythroblasts to the late mature polychromatic erythroblasts, most reaching the lowest numbers after 7 days of infection, and again followed by gradual recoveries. This was also accompanied by late increases in the expression of hemoglobin, in both the spleen and BM.

Iron is essential not only for the host but also for pathogen proliferation, and trypanosome infections are no exception.[17,18] In the case of extracellular, blood circulating pathogens, iron is rapidly removed from circulation to prevent pathogens from accessing it. This is seen in BALB/c and C57BL/6 mice by the expression profiles of transferrin and ferritin, particularly in the liver, where early increases in both would facilitate iron retention and removal from circulation, as well as by decreases in circulating serum iron and transferrin saturation, and an increase in circulating ferritin.[33] This response might be particularly important in the earliest days of infection, since *T. brucei* bloodstream forms can acquire iron through pathogen-specific receptors for transferrin and haptoglobin-hemoglobin complexes.[34] On the other hand, the higher levels of serum iron and circulating ferritin levels observed in *Hamp*$^{-/-}$ mice do not appear to influence pathogen proliferation, when compared with the wild-type animals, with parasite levels remaining similar. Although limited data are available regarding extracellular and intracellular parasites, they can both benefit or be suppressed by high iron levels.[27,28] This is in contrast with many bacterial infections, where hepcidin is known to have a crucial role in creating a hypoferrimic state, to limit iron availability, and protect the host both against infections caused by siderophilic bacteria[23,35]

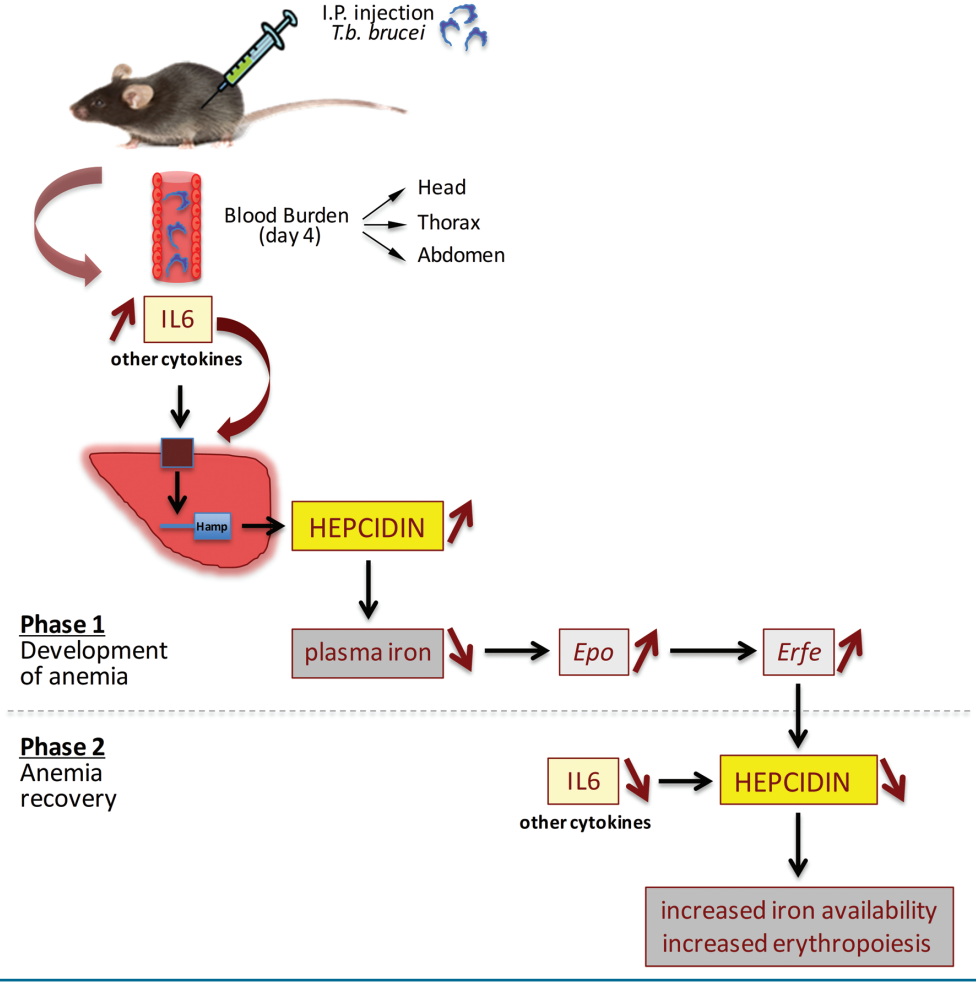

Figure 8. Schematic representation of the mechanisms of hepcidin regulation during the development and recovery from anemia in *T.b. brucei* infections.

and by others.[36] Additionally, there is an increase in iron storage both in the liver, the major organ for iron accumulation, and the spleen, where iron recycling from senescent erythrocytes occurs. However, this redistribution of iron with the goal of limiting its mobilization and availability to pathogens is actually a double-edged sword; at the same time potentially limiting iron availability for erythropoiesis in the BM and leading to the condition known as anemia of inflammation,[37,38] thus contributing to the overall trypanosome-related anemia.

On the inflammatory side, it has been well documented that the response to infectious/inflammatory stimuli involves the expression of numerous pro- and anti-inflammatory cytokines that have various effects on different leukocyte populations, from lymphocytes to macrophages, with the latter also being involved in the modulation of iron homeostasis. We evaluated the levels of some relevant circulating inflammatory cytokines, where we observed a strong type I cytokine response in all models, with increases in the levels of IL-6, IFN-γ and TNF-α. IL-6, which is mostly produced by macrophages but also by Th2 T cells in response to the extracellular parasites, is a major inducer of hepcidin expression by the liver during inflammatory processes.[37,40,41] In trypanosomiasis, increased levels of IFN-γ can inhibit BM proliferation and suppress erythropoiesis,[42] whereas TNF-α is known to be a key mediator involved in parasitemia control but can also contribute to enhanced erythrophagocytosis.[43,44] Furthermore, these cytokines favor the maturation of naïve T cells into Th1 T cells, which are involved in cell-mediated immunity. We also observed extremely high levels of MCP1, a chemokine that plays an important role in monocyte recruitment.[45] Contrary to other protozoan infections, such as those from *Leishmania major*,[46] *Toxoplasma gondii*[47] or *Plasmodium chabaudi*,[48] where this recruitment is essential for the effective control of the infection, in *T. brucei* infections expression of MCP1 and other chemokines seem to have deleterious effects, especially during early infection, contributing to enhanced pathogenesis.[49,50] However, such effects might be mitigated by the production of the type II cytokine IL-10, which potentially limits MCP1 expression and reduces monocyte recruitment from the BM.[51] IL-10 is also known to down-regulate IFN-γ and TNF-α, and, depending on the balance between these cytokines, it may contribute to attenuate the severity of the anemia.[52]

During the development of the immune response to various pathogens, hepcidin is known to be key in the regulation of iron metabolism, leading to reduced mobilization and redistribution of iron in order to limit its access by pathogens, and in turn, to the so-called anemia of inflammation.[37,38] However, there are cases where iron redistribution and anemia occur but by mechanisms that are hepcidin-independent.[22] As such, we investigated the possible role of hepcidin in the development of trypanosome-related anemia and further looked into the molecular mechanisms subjacent to the transition from a status of acute anemia to a status of recovery/chronic anemia.

Increases in hepcidin expression were observed in BALB/c and C57BL/6 mice, with no discernible expression in *Hamp*$^{-/-}$ mice. The liver is long known to be the major contributor for systemic hepcidin levels, and thus the master regulator of iron homeostasis. In response to an infectious/inflammatory stimulus, an increased expression of hepcidin is triggered in the liver, mostly mediated by IL-6. Hepcidin then binds to ferroportin, leading to its internalization and degradation, effectively blocking iron release from hepatocytes, intestinal enterocytes and macrophages.[19-21,37,53] In prolonged infections, this limits iron availability for the pathogens, but also for the host itself, thus leading to the aforementioned anemia of inflammation. However, since there is no hepcidin in *Hamp*$^{-/-}$ mice, there is no limitation in iron availability, so the milder anemia observed in these animals is likely mediated by hepcidin-independent mechanisms, which is not always required for the onset of early inflammatory hypoferremia.[54,55]

The increased hepcidin expression in the spleen, kidney and BM is expected to have a low impact on systemic iron homeostasis, but may have an important role in the control of local iron fluxes. As with the hematologic parameters, there is a turning point at around day 7 when the infectious stimulus that leads to increased hepcidin expression seems to be replaced by an inhibitory signal that suppresses hepcidin. This could partially be explained by a decrease in IL-6 levels, but there are likely other signaling pathways contributing to this suppression. As such, we also evaluated the expression of genes that are influenced by hepcidin or, in turn, influence hepcidin expression.

Ferroportin is the major target for hepcidin, being removed from the cell surface and also inhibited at the expression level.[21,56] As the sole known iron exporter, this interaction will severely limit iron release and mobilization, especially by the intestinal enterocytes, recycling macrophages and hepatocytes, leading to hypoferremia. In both BALB/c and C57BL/6 mice, ferroportin expression correlates both negatively with hepcidin expression and positively with the development of anemia, being down-regulated at the earlier days of infection. This limits iron release for the production of new erythrocytes and leads to anemia. It is subsequently up-regulated over the following days when iron is again being released and enhanced erythropoiesis occurs, allowing a recovery from anemia. However, in *Hamp*$^{-/-}$ mice there is no such control of ferroportin due to the lack of hepcidin, so iron is readily available to allow for the faster recovery from anemia observed in these animals. A similar regulation of ferroportin was observed at the protein level. Levels in the liver of C57BL/6 mice closely matched variations in mRNA expression, and also mirrored hepcidin expression, with a decrease up to day 7. This is then followed by a recovery and an increase over subsequent days, indicating an early iron retention and a later release from the liver. In *Hamp*$^{-/-}$ mice, liver FPN1 levels also closely matched mRNA expression, and were kept elevated throughout the experiment, with the zenith at day 7. These results show that there was no limitation in iron release from the liver during infection, thus supporting the hypothesis of a faster erythrocyte recovery when compared with C57BL/6 mice. In the spleen, a similar response was observed for both C57BL/6 and *Hamp*$^{-/-}$, with a very significant decrease in ferroportin levels, followed by a later increase in the second stage of infection, albeit faster and higher in the *Hamp*$^{-/-}$ mice. During anemia of inflammation, after erythrophagocytosis, iron is not properly released from macrophages due to ferroportin internalization mediated by hepcidin (hence the development of anemia). But at a later stage, during recovery, ferroportin levels are normalized, iron mobilized and erythropoiesis also normalizes,

leading to a recovery from anemia. No significant changes were observed in duodenal levels of ferroportin. It is likely that body iron levels were already sufficient to cope with the erythropoietic demands, so there was no need for additional dietary iron absorption. Additionally, we must also consider that analysis of ferroportin by Western blot does not distinguish between functional ferroportin on the cell membrane and possibly non-functional ferroportin in intracellular compartments, which could hide the smaller differences between WT and KO mice. Nevertheless, it is clear that the lack of hepcidin allows for a faster recovery and normalization of ferroportin levels, and thus, for an earlier availability of iron required for erythropoiesis.

The later suppression of hepcidin also negatively correlates with increases in the expression of several erythroid regulators. Erythropoietin (EPO) is one of the signaling molecules driving erythropoiesis, being produced mostly by the kidney. It is essential for EPO receptor (R)-mediated erythropoiesis that occurs in the BM and the spleen. Although EPO can influence hepcidin expression, it does not seem to act directly on it, but rather indirectly through erythroferrone (ERFE) produced by erythroid progenitors.[57] Interestingly, there is no major role for ERFE in baseline erythropoiesis, but it rather functions during erythropoiesis-related stress[58] and during recovery from anemia of inflammation,[59] by suppressing hepcidin and increasing iron availability. Our data show that in *Hamp*$^{-/-}$ mice ERFE does not seem to be involved in the recovery from anemia, despite the increase in EPO, since no variations in expression were observed, which opens up the possibility that ERFE is not only involved in hepcidin suppression, but also acts as a sensor for hepcidin levels. Other erythroid regulators that can influence hepcidin, such as the predominantly erythroblast-produced GDF15 and TWSG1,[60,61] were also found to be over-expressed at the later stage of infection and could contribute to hepcidin suppression. These findings are very similar to previous observations in bacterial infections. During injection with heat-inactivated *Brucella abortus*,[25,26] C57BL/6 mice have similar patterns of hepcidin expression, with a significant increase in the early days and a decrease in later days of infection. Mice also develop anemia of inflammation and iron restriction, and can only partially recover from it. However, when hepcidin is suppressed (in *Hamp*$^{-/-}$ mice), anemia is ameliorated and there is a faster recovery. Furthermore, a role for IL-6 in the onset and resolution of anemia is also shown,[26] both by triggering increased hepcidin expression and by interfering with erythropoiesis. However, recovery from anemia in IL-6$^{-/-}$ mice is not as fast as in *Hamp*$^{-/-}$ mice, showing that although IL-6 is a strong inducer of hepcidin during inflammatory conditions, it is not the only one.

In summary, *T.b. brucei* infection leads to the rapid development of anemia followed by a partial recovery (Figure 8). In the acute phase, a strong inflammatory signature is associated with hepcidin expression causing iron redistribution and limited availability. During the recovery phase, the decrease in hepcidin expression might be due to the decrease in the inflammatory response and the increased production of erythroid regulators. Importantly, the lack of hepcidin clearly reduces the severity of trypanosome-derived anemia. This knowledge could contribute to the development of novel strategies for the treatment and control of trypanosomiasis-derived anemia, limiting its impact on human and non-human health.

Disclosures

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Contributions

JVN, ACG, DMC and CB performed research; JVN, ACG and DMC performed data analysis; JVN, ACG and DMC wrote the manuscript; JVN, JT, ACS and PNSR supervised the study; SV provided the Hamp KO animals; PNSR, JT, ACS, SV contributed data and edited the manuscript. All authors revised and approved the manuscript.

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