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

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**Running title**

Hepcidin and *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 hepcidin elevation declines due to the decreased 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, in hepcidin knockout mice, anaemia is much milder and its recovery is complete, in contrast to wild-type animals which do not fully recover from anemia after 21 days. Aside from all other factors known to be involved in the development of anemia during trypanosomiasis, there clearly is an important contribution of hepcidin for both its development and recovery.


Introduction

African trypanosomes are extracellular protozoan parasites transmitted by the hematophagous tsetse fly (*Glossina spp*) and 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 affects mostly cattle\(^2\).

One of the most common complications derived from 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 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 extracellular 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, 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 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\cite{22-26} and some intracellular parasites\cite{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\cite{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.

**Hematological and serum parameters, tissue iron content.** Hematological 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\cite{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 cells were stained with anti-CD3e (17A2), anti-CD19 (6D5), anti-TER119, anti-CD71 (RI7217), anti-CD11c (N418), and anti-CD11b (M1/70) antibodies, run in a BD FACSCanto II Flow Cytometer (BD Biosciences) and data 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, Lisboa, Portugal).

**Analysis of gene expression by qPCR.** Relative levels of several genes mRNAs were quantified in relevant organs of control and infected animals, by 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 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. A $p$ value of less than 0.05 was considered statistically significant.

Further details of the study methods are included in supplemental information.
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 (Supplementary Figure 1). 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 of the mean corpuscular volume (MCV) was also observed, indicative of macrocytic anemia (Figure 1E). Alterations in the development of erythroid lineage in the bone marrow 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 1I).

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 1O).

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-inflammatory 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-gamma, TNF-alpha 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-gamma and IL-10 returning to normal levels at 21 days post-infection. Additionally, high levels of MCP-1 were observed as early as 1-day post-infection, remaining high at 4 days 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 bone marrow. 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 downregulated 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 upregulated 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,M).

In the spleen, *Hamp1* was observed to be upregulated 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 kept elevated up to day 21.
In the kidney, an increase in *Hamp1* expression was observed starting day 1, peaking at day 4, and returning to control levels at day 7 (Figure 3L). Levels of *Epo* were upregulated throughout the duration of the experiment (Figure 3M).

Finally, in the bone marrow, *Hamp1* expression was found to be increased as early as day 1, followed by a gradual downregulation 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 3O-Q). *Gdf15* and *Twsg1* expressions gradually increased throughout the course of the infection, reaching maximum levels at day 21 (Figure 3R,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 (*Hamp1*-/-) mice. No significant differences were found in the parasitemias or total parasite burdens between *Hamp1*-/- and C57BL/6 mice (Supplementary Figure 2). Hematological 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 *Hamp1*-/- mice there is a 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,D). A significant increase of reticulocyte numbers and MCV was also observed at later stages of infection, in both wild-type and knockout mice (Figure 4B,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,G), increases in TIBC (Figure 4H), as well as
liver and spleen iron (Figure 4J,K, Supplementary Figure 3), with no changes in ferritin levels (Figure 4I). In Hamp−/− mice, only a similar increase in TIBC (Figure 4H) was observed, as well as increases in liver and spleen iron (Figure 4J,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,I).

Circulating cytokine levels in both C57BL/6 and Hamp−/− mice were mostly comparable to BALB/c. IL-6 levels increased as early as 1-day post-infection and remained high up to 7 or 14 days (in C57BL/6 and Hamp−/−), with a subsequent gradual recovery to control levels (Figure 5A). Circulating levels of IFN-gamma, TNF-alpha 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 1-day post-infection, reaching maximum levels at 4 days and rapidly decreasing to normal levels at 7 days 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,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 ferroportin expression was kept elevated throughout the duration of the experiment (Figure 6A,B). In the spleen, an increase of Hbb (Figure 6C) and Twsg1 (Figure 6E) was observed in both wild-type and 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 upregulated 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 (Figure 7A), 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−/−. In the spleen (Figure 7B), ferroportin levels gradually decreased until being undetected 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 and with much higher levels at day 21. No significant changes in ferroportin protein levels were observed in the duodenum (Figure 7C).

Discussion

In this work, using a mouse model of trypanosomiasis, which has been shown to replicate the two stages of the disease occurring in humans, we have performed an integrated analysis 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, to the anemia established during T. b. brucei infection. Different degrees of anemia severity in trypanosomal infections have been reported and described to be dependent on the host and sub-species causing the infection. In this study an early decrease in several hematological parameters such as the number of RBC and reticulocytes, hematocrit and hemoglobin levels, and an increase of the 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 the increase of most parameters,
never reaching normal levels in wild-type (BALB/c and C57BL/6) animals but fully recovering in Hamp\(^{-/-}\) mice.

An in depth look at several erythrocyte populations in the bone marrow of BALB/c mice shows similar patterns in all of them, from the early immature pro-erythroblasts to the late mature polychromatric erythroblasts reaching the lowest numbers mostly 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 bone marrow.

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 don't appear to influence pathogen proliferation, when compared with wild-type animal, with parasite levels remaining similar. Although limited data is 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 an hypoferrimic state, to limit iron availability and protect the host against infections caused not only by siderophilic bacteria\(^{23,35}\), but also 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 bone marrow and leading to the condition known as anemia of inflammation\textsuperscript{37,38}, hence contributing to the overall trypanosome-related anemia.

On the inflammatory side, it is 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\textsuperscript{39}. 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-gamma and TNF-alpha. 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\textsuperscript{37,40,41}. In trypanosomiasis, elevated levels of IFN-gamma can inhibit bone marrow proliferation and suppress erythropoiesis\textsuperscript{42}, whereas TNF-alpha is known to be a key mediator involved in parasitemia control but it can also contribute to enhanced erythrophagocytosis\textsuperscript{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\textsuperscript{45}. Contrary to other protozoan infections, such as with \textit{Leishmania major}\textsuperscript{46}, \textit{Toxoplasma gondii}\textsuperscript{47} or \textit{Plasmodium chabaudi}\textsuperscript{48}, where this recruitment is essential for the effective control of the infection, in \textit{T. brucei} infections expression of MCP1 and other chemokines seem to have deleterious effects, especially during early infection, contributing to enhanced pathogenesis\textsuperscript{49,50}. However, this deleterious effect might be mitigated by the production of the type II cytokine IL-10, which potentially limits MCP1 expression and reduces monocyte recruitment from the bone marrow\textsuperscript{51}. IL-10 is also known to downregulate IFN-gamma and
TNF-alpha, and depending on the balance between these cytokines, it may contribute to attenuate the severity of the anemia.\textsuperscript{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.\textsuperscript{37,38} However, there are cases where iron redistribution and anemia occur but by mechanisms that are hepcidin-independent.\textsuperscript{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 \textit{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.\textsuperscript{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 \textit{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.\textsuperscript{54,55}

The increased hepcidin expression in the spleen, kidney and bone marrow is expected to have a low impact on systemic iron homeostasis, but may have an important role in the control of local iron fluxes. Just like with the hematological parameters, there is a turning point at around day 7, where 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 downregulated at the earlier days of infection, thus limiting iron release for the production of new erythrocytes and leading to anemia, and upregulated on the later days, when iron is again being released and enhanced erythropoiesis occurs, allowing a recovery from anemia. However, in \(\text{Hamp}^{-/-}\) 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 followed by a recovery and increase at later days, indicating an early iron retention and a later release from the liver. In \(\text{Hamp}^{-/-}\) mice, liver FPN1 levels also closely matched mRNA expression, and were kept elevated throughout the experiment, with the zenith at 7 days. 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 \(\text{Hamp}^{-/-}\), 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 \(\text{Hamp}^{-/-}\) 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 nonfunctional ferroportin in intracellular compartments, thus possibly hiding 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. EPO is one of the signaling molecules driving erythropoiesis, being produced mostly by the kidney, and is essential for EPOR-mediated erythropoiesis that occurs in the bone marrow and the spleen. Although EPO can influence hepcidin expression, it does not seem to act directly on it, but rather indirectly through ERFE, produced by erythroid progenitors. Interestingly, there isn’t a major role for ERFE in baseline erythropoiesis, but it instead functions during erythropoiesis-related stress and during recovery from anemia of inflammation, 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 the possibility for ERFE not only being involved in hepcidin suppression, but also acting as a sensor for hepcidin levels. Other erythroid regulators that can influence hepcidin, such as the predominantly erythroblast-produced
GDF15 and TWSG1\textsuperscript{60,61}, were also found to be overexpressed at the later stage of infection and could contribute to hepcidin suppression. These findings are very similar to what was previously observed for bacterial infections. During injection with heat-inactivated \textit{Brucella abortus}\textsuperscript{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 \textit{Hamp}/\textsuperscript{-} 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\textsuperscript{26}, by triggering increased hepcidin expression, but also by interfering with erythropoiesis. However, recovery from anemia in \textit{IL-6}/\textsuperscript{-} mice is not as fast as in \textit{Hamp}/\textsuperscript{-} mice, showing that although IL-6 is a strong inducer of hepcidin during inflammatory conditions, it is not the only one.

In summary, \textit{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 on hepcidin expression might be due to the decrease on 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 impacts on human and non-human health.
References


**Figure 1** – Hematological parameters, bone marrow 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*. Hematological parameters were blindly determined by a certified laboratory and bone marrow cells were isolated, stained with antibodies for erythroid populations and analysed 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, (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, (K) transferrin saturation (TSAT), (L) total iron binding capacity (TIBC), (M) serum ferritin, (N) total liver iron, (O) total spleen iron. Values are represented as means ± standard deviation (n=5). Differences from the control groups were considered statistically significant at *p<0.05, **p<0.01, and ***p<0.001.

**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-gamma, (C) TNF-alpha, (D) IL-10 and (E) MCP1 circulating levels, using a Cytometric Bead Array (CBA) Mouse Inflammation Kit. Values are represented as means ± standard deviation (n=5). Differences from the control groups were considered significant at *p<0.05, **p<0.01, and ***p<0.001.

**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 PCR. **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 means ± standard deviation (n=5). Differences from the control groups were considered significant at *p<0.05, **p<0.01, and ***p<0.001.

Figure 4 – Hematological 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. Hematological 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), (FE) serum iron, (G) transferrin saturation (TSAT), (H) total iron binding capacity (TIBC), (I) serum ferritin, (J) total liver iron, (K) total spleen iron. 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.

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-gamma, (C) TNF-alpha, (D) IL-10 and (E) MCP1 circulating levels, using a Cytometric Bead Array (CBA) Mouse Inflammation Kit. 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.
Figure 6 – Gene expression in the liver, spleen and kidney of C57BL/6 and Hamp\textsuperscript{−/−} mice after experimental infection with \textit{T.b. brucei}. Relative mRNA expression of several genes was measured 1, 4, 7, 14, and 21 days post-infection, by real-time PCR. (A) liver \textit{Hamp1}, (B) liver \textit{FPn1}, (C) spleen \textit{Hbb}, (D) spleen \textit{Erfe}, (E) spleen \textit{Twsg1}, (F) kidney \textit{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\textsuperscript{−/−} mice, \(g\), \(h\), \(i\) between infected groups and \(j\), \(k\), \(l\) between control groups.

Figure 7 – Ferroportin protein levels in the liver, spleen and duodenum of C57BL/6 and Hamp\textsuperscript{−/−} mice during infection \textit{T.b. brucei}, analysed 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 means ± 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\textsuperscript{−/−} mice and \(g\), \(h\), \(i\) between C57BL/6 control and Hamp\textsuperscript{−/−} mice. NI - non-infected; dpi - days post-infection.

Figure 8 – Schematic representation of the mechanisms of hepcidin regulation during the development and recovery from anemia in \textit{T.b. brucei} infections.
I.P. injection *T. b. brucei*

Blood Burden (day 4)

- Head
- Thorax
- Abdomen

**Phase 1**
Development of anemia

- IL6
- other cytokines

- Hamp

- HEPCIDIN

- plasma iron

- Epo

- Erfe

**Phase 2**
Anemia recovery

- IL6
- other cytokines

- HEPCIDIN

- increased iron availability

- increased erythropoiesis
Methods

Mice, Parasites, Infections and Sampling

All animals were housed in the animal facility of the Instituto de Investigação e Inovação em Saúde (i3S), kept in individually ventilated cages with high efficiency particulate air (HEPA) filters and were fed ad libitum with sterilized food and water. 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. Mice were infected by intraperitoneal injection with 100 µl containing $10^5$ blood parasites harvested at the first peak of parasitemia from a donor mouse. Control animals were similarly injected with the same volume of PBS. Parasitemia was assessed with a hemocytometer, using blood collected by tail vein puncture, diluted in equal parts with Ethylenediaminetetraacetic acid (EDTA) and further diluted to the appropriate density in 0.85% ammonium chloride to enable erythrocyte lysis. The detection limit achieved was $1 \times 10^5$ parasites/ml. Parasite loads were assessed using the IVIS Lumina. *T. b. brucei* GVR35-infected mice were anesthetized with 2.5% isoflurane (O$_2$ flow of 1 L/min). D-luciferin (100 mg/Kg, Perkin Elmer, Waltham MA, USA) was administered subcutaneously in the neck five minutes prior to image acquisition. Mice were then transferred to the stage of an intensified charge-coupled device photon-counting video camera box where anesthesia was maintained with 2.5% isoflurane (O$_2$ flow of 0.3 L/min). Exposure to isoflurane was standardized among groups and throughout all time points and signal acquisition was controlled by the Living Image software (Perkin Elmer). The detection of the bioluminescence signal by the system resulted in the generation of signal maps automatically superimposed to the grey-scale photograph of the mice. The regions of interest (ROI) encompassing most of the ventral view of the animal body, the head, the thorax and the abdomen were manually defined. The quantifications were performed using the Living Image software (Perkin Elmer). The total flux (photons/second)
and average radiance (photons/second/cm²/steradian) within these ROIs were automatically calculated. The percentage of bioluminescence signal in the defined regions was calculated by dividing the total flux of the respective ROI by the total flux of the ventral animal body ROI. After imaging, blood was collected while the animals were still anesthetized, after which they were sacrificed by cervical dislocation. For each experimental animal, liver, spleen, kidney and bone marrow from femur were collected, weighted and immediately frozen in liquid nitrogen and stored at -80ºC until further use.

**Hematological parameters, serum parameters and tissue iron content.** For determination of hematological parameters (red blood cells, reticulocytes, hemoglobin, hematocrit, mean corpuscular volume), 100 µl of blood were used in 1:1 dilutions with EDTA (1:10 diluted in sterile PBS) (BD Biosciences, San Jose CA, USA). For determination of serum parameters (serum iron, serum ferritin, transferrin saturation, unsaturated iron binding capacity, total iron binding capacity), non-heparinized blood was transferred into 1.5 ml microcentrifuge tubes, allowed to clot for 4 h at 4ºC, and centrifuged at 16000×g until a clear serum was obtained. For non-heme iron measurements, briefly, tissue samples with an average weight of 50 mg were placed in iron-free Teflon vessels (ACV-Advanced Composite Vessel, CEM Corporation, Matthews NC, USA) and dried in a microwave oven (MDS 2000, CEM Corporation). Subsequently, dry tissue weights were determined and samples digested in an acid mixture (30% hydrochloric acid and 10% trichloroacetic acid) for 20 h at 65ºC. After digestion, a chromogen reagent (5 volumes of deionised water, 5 volumes of saturated sodium acetate and 1 volume of 0.1% bathophenanthroline sulfonate/1% thioglycollic acid) was added to the samples in order to react with iron and obtain a colored product that was measured spectrophotometrically at 535 nm. The extinction coefficient for bathophenanthroline is 22.14 mM⁻¹cm⁻¹.
**Cytokine profile analysis.** The BD Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Biosciences, San Jose CA, USA) allows the simultaneous quantification of Interleukin(IL)-6, IL-10, Tumour Necrosis Factor(TNF)-alpha, Interferon(IFN)-gamma and Monocyte Chemoattractant Protein(MCP) 1. Briefly, serum samples were diluted 1:5 v/v in Assay Diluent, and plated in 96-well plates with mixed Capture Beads and PE Detection Reagent. The mixture was incubated for 2 h at RT in the dark, after which wash buffer was added prior to centrifugation for 1 min at 200×g. The supernatant was discarded and the beads resuspended in wash buffer. Samples were run in a BD FACSCanto II Flow Cytometer and results were analysed with the FCAP Array Software (BD Biosciences).

**Flow cytometry.** Bone marrow cells were isolated by flushing long bones in DMEM supplemented with 2% heat-inactivated fetal bovine serum, L-Glutamine and PenStrep (all from Thermo Fisher Scientific, Waltham MA, USA). Cells were counted using the trypan blue exclusion assay. A million bone marrow cells were plated and stained with anti-CD3e (17A2), anti-CD19 (6D5), anti-TER119, anti-CD71 (RI7217), anti-CD11c (N418), and anti-CD11b (M1/70) antibodies for 30 min on ice. All antibodies were obtained from BioLegend (San Diego CA, USA). Cells were then washed and fixed with 2% PFA for 10 min at room temperature. Cells were run in a BD FACSCanto II Flow Cytometer (BD Biosciences) and data was analyzed with FlowJo software (FlowJo LCC, Ashland OR, USA). Erythroblast populations were gated based on the expression of the surface markers TER119 and transferrin receptor 1 (CD71) into pro-erythroblasts, basophilic and polychromatic erythroblasts.

**Analysis of gene expression by quantitative RT-PCR.**

Gene expression was analysed using a CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules CA, USA). Genes analyzed include genes involved in iron homeostasis and

One µl of each cDNA sample was added to a reaction mix containing 10 µl iQ SYBR Green Supermix (Bio-Rad), 7 µl of ddH2O and 250 nM of each primer (Supplementary Table 1), making a total volume of 20 µl per reaction. A non-template control was included for each set of primers. The cycling profile was as follows: 95ºC for 3.5 min, 40 cycles of 95ºC for 20 s and 59ºC for 20 s. Samples were prepared in duplicates, a melting curve was generated for every PCR product to confirm the specificity of the assays and a dilution series was prepared to check the efficiency of the reactions. Messenger RNA expression was normalized to Hypoxanthine Guanine Phosphoribosyltranferase 1 (*Hprt*) mRNA.

**Analysis of ferroportin protein levels by Western blot.** *Protein extraction.* Tissues were homogenized with a tissue grinder in RIPA buffer (3 µl/mg of tissue), supplemented with 10 µl PMSF 100 mM, 10 µl protease inhibitors and 1 µl DTT 1M per ml of buffer, and placed on ice for 20 min. Samples were then centrifuged for 20 min at 14000×g at 4ºC, and supernatants collected. Protein concentration was determined with the Bio-rad DC Protein Assay (Bio-Rad, Hercules CA, USA). *Western blot.* Protein extracts were prepared in Laemmli buffer (Bio-rad). Equivalent amounts of protein (30 µg) were separated by electrophoresis in 12% SDS-polyacrylamide gels (SDS-PAGE) and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane for 90 min at 100 V. After blocking the membrane with 5% BSA in TBST (50 mM Tris-HCl, pH 8; 154 mM NaCl; 0.1% Tween 20) overnight at 4ºC, membranes were incubated with the primary antibody following the manufacturer’s instructions: rabbit anti-FPN (Ferroportin/SLC40A1 Antibody from Novus, Littletown, CO, USA catalog #NPB1-21502), (1:1000) 1 h RT, rabbit anti-GAPDH
(Abcam, Cambridge, UK, catalog #EPR16891), (1:1000) 1 h RT. Membranes were washed and incubated with the secondary anti-rabbit (1:10000) in 1% BSA in TBST. Membranes were then incubated with the Luminata Crescendo Western HRP substrate, imaged with ChemiDoc imaging system (Bio-Rad) and analysed in ImageLab software (Bio-Rad).

**Histological analysis.** Sections of liver and spleen were fixed in 10% neutral buffered formalin (Bio-Optica, Milan, Italy) at 4°C for 8 h. After dehydration, sections were embedded in paraffin, sectioned at 3 μm, and stained with Perl's Prussian Blue for ferric iron visualization. Control sections were also stained with hematoxylin and eosin (H&E).

**Results**

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

The bioluminescence signal was detectable at day 1 and when parasites were still undetectable by microscopic analysis of peripheral blood (Supplementary Figure 1A,B). Once parasite burden increased, bioluminescence was detected, as expected, along the entire body of the animal (day 4). Hotspot signal appearing in the head region and indicative of infection of the central nervous system was visible at day 7 (Supplementary Figure 1A). Parasitemias reached a peak at day 4 and after a slight decrease on day 7 were maintained at relatively steady values close to 10⁸ parasites/ml of blood (Supplementary Figure 1B). As expected, both methodologies show comparable progression of parasite burden, with the exception of day 1, when parasites were still undetectable by microscopic analysis of peripheral blood. To study the dynamics of parasite distribution and dissemination, the images obtained for each animal were segmented into several areas corresponding to the whole body, head, thoracic region and abdomen (Supplementary Figure 1C). This analysis showed a progressive increase on the percentage of the bioluminescent signal in the head
of the animals peaking at day 7 (Supplementary Figure 1C). From day 14, most of the bioluminescence signal detected corresponds to the abdominal region, which might be due to colonization of tissues such as the adipose tissue\textsuperscript{31}. 
**Supplementary Table 1.** Primers used for gene expression analysis.

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Supplementary Figure 1 - Parasite burdens during the course of infection with *T. b. brucei* in BALB/c mice. (A) Female BALB/c mice infected with 10^5 luciferase-expressing *T. b. brucei* BSFs by intraperitoneal injection. Whole-mouse bioluminescence imaging was performed 1, 4, 7, 14, and 21 days post-infection. Images of infected animals are shown and the bioluminescence expressed in average radiance (photons per seconds per square centimeter per steradian [p/sec/cm^2/sr]) was quantified. (B) Parasitemia of infected animals. The detection limit was 1×10^5 parasites/ml. n.d. not detected. (C) Percentage of bioluminescence signal throughout the infectious process in different regions of interest (ROIs), namely head, thorax and abdomen. Representative images containing the defined ROIs are shown. Values are represented as means ± standard deviation (n=5). Differences between days post-infection were considered significant at *p<0.05, **p<0.001* and *p<0.0001*. 
Supplementary Figure 2 - Parasite distribution during the course of infection with T. b. brucei in C57BL/6 and Hamp⁻/⁻ mice. (A) Female C57BL/6 and Hamp⁻/⁻ mice infected with 10⁵ luciferase-expressing T. b. brucei BSFs by intraperitoneal injection. Whole-mouse bioluminescence imaging was performed 1, 4, 7, 14, and 21 days post-infection. (B) Quantification of bioluminescence expressed in average radiance (photons per seconds per square centimeter per steradian [p/sec/cm²/sr]). (C) Parasitemia of infected animals. On day 7 post-infection only one animal in each group had detectable parasites in the blood. The detection limit was 1×10⁵ parasites/ml. n.d. not detected.
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Supplementary Figure 3 – Tissue iron content evaluated by histological analysis, with Perl’s method for iron staining. Iron content was evaluated in the liver and spleen of uninfected and infected C57BL6 and Hamp−/− mice, at 0, 1, 4, 7, 14, and 21 days post infection (dpi). Higher basal levels could be observed in both the liver and spleen of Hamp−/− mice, as well as a continuous deposition in both of infected C57BL/6 and Hamp−/− mice, but clearly more noticeable in the later. Blue staining denotes accumulation of ferric iron.