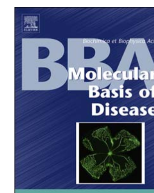
BBA - Molecular Basis of Disease 1863 (2017) 3049–3059

Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

journal homepage: www.elsevier.com/locate/bbadis



Iron supplementation in mouse expands cellular innate defences in spleen and defers lethal malaria infection

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A R T I C L E I N F O

Keywords:
Iron supplementation
Oxidative stress
Malaria
Immune response
Hemochromatosis

A B S T R A C T

The co-endemicity of malnutrition, erythrocytopathies, transmissible diseases and iron-deficiency contribute to the prevalence of chronic anaemia in many populations of the developing world. Although iron dietary supplementation is applied or recommended in at risk populations, its use is controversial due to undesirable outcomes, particularly regarding the response to infections, including highly prevalent malaria. We hypothesized that a boosted oxidative stress due to iron supplementation have a similar impact on malaria to that of hereditary anaemias, enhancing innate response and conditioning tissues to prevent damage during infection. Thus, we have analysed antioxidant and innate responses against lethal *Plasmodium yoelii* during the first five days of infection in an iron-supplemented mouse. This murine model showed high iron concentration in plasma with upregulated expression of hemoxygenase-1. The sustained homeostasis after this extrinsic iron conditioning, delayed parasitemia growth that, once installed, developed without anaemia. This protection was not conferred by the intrinsic iron overload of hereditary hemochromatosis. Upon iron-supplementation, a large increase of the macrophages/dendritic cells ratio and the antigen presenting cells was observed in the mouse spleen, independently of malaria infection. Complementary, malaria promoted the splenic B and T CD4 cells activation. Our results show that the iron supplementation in mice prepares host tissues for oxidative-stress and induces unspecific cellular immune responses, which could be seen as an advantage to promote early defences against malaria infection.

1. Introduction

Anaemia affects 1.62 billion people globally, with a prevalence of 43% in developing countries of Africa and Asia. More than 85% of the world's anaemia burden happens in the 3 high-risk groups of children under 5 years old, pregnant women and non-pregnant women aged 15–49 [1]. Half of the anaemia cases are due to iron deficiency, the most common and widespread nutritional disorder in the world [2] arising when total body iron stores are low or when the iron supply to the bone marrow is inadequate [3]. Iron, is the essential trace mineral in the haemoglobin and myoglobin structures but also plays a critical role in the interaction between host and pathogens. Indeed, the control of iron distribution in organs and cells is by itself an innate immune mechanism against the invading microorganisms [4,5]. For instance, the peptide hepcidin, a key mammalian inhibitor of the iron entry into serum, is induced by interleukins during infection and inflammation [4,6,7]. Besides, some myeloid cell types express hepcidin mRNA in response to pathogens through the specific activation of the toll-like receptor 4 [8].

To prevent iron deficiency anaemia in at-risk populations and to treat patients with proven disease, iron dietary supplementation is routinely used [3]. Nevertheless, there is a controversy on the impact of

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http://dx.doi.org/10.1016/j.bbadis.2017.09.027
Received 3 August 2017; Received in revised form 26 September 2017; Accepted 27 September 2017
Available online 29 September 2017
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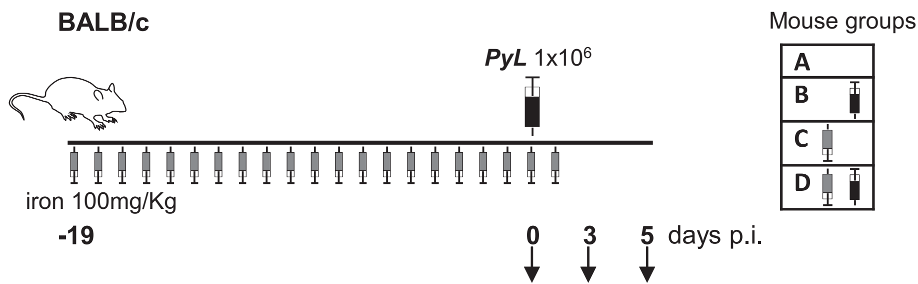

Fig. 1. Experimental design used to study a lethal malaria infection in iron overloaded mice. BALB/c mice were inoculated with iron-dextran 100 mg/kg/day from day − 19 to + 1 p.i. Mice were i.p. infected with 10^6 *PyL*-iRBCs on day 0 p.i. According to the treatment, mice were divided in the following groups: A) control healthy mice; B) infected mice; C) iron supplemented mice; and D) infected iron supplemented mice. Random mice from each group were sacrificed on days 1, 3 and 5 p.i. to perform the described analyses.

iron supplementation [9–12], particularly with respect to apply it in regions where endemicity of iron deficiency coexists with high frequency of carriers of hereditary erythrocytopathies (as HbS or G6PD deficiency) and several other transmissible diseases causing anaemia as malaria. Iron supplementation in malaria endemic populations has been reported to increase malaria risk [5,13–15] or not modify it [16–21]. Malaria itself causes profound disturbance of iron distribution as a consequence of haemolysis, heme release, dyserythropoiesis, anaemia, iron deposition in macrophages, inhibition of dietary iron absorption and immune response [22]. In addition, frequent malnutrition, erythrocytopathies and other co-infections in iron-deficiency/malaria endemic regions contribute to sustain anaemia prevalence in the affected populations [23].

On the other hand, the association of anaemia and malaria is not always detrimental. Hence, common haemoglobinopathies causing anaemia, as the sickle cell trait (heterozygous HbS), can confer significant degrees of protection against severe, life-threatening *Plasmodium falciparum* malaria [24–27]. The mechanism of this protection in humans is based on accumulating oxidized and denatured haemoglobin [28,29]. Moreover, during malaria infection, mouse models bearing HbS have shown to upregulate the detoxifying enzyme hemoxygenase-1 (HO-1), thus preventing the cytotoxic accumulation of the free heme [30,31]. This evidence shows an association between levels of tolerated anaemia with an induced antioxidant capacity to promote innate immune responses that could determine the outcome of *Plasmodium* infection. Moreover, tolerance to experimental infection in mice by *P. chabaudi* is associated to the expression of ferritin H chain as stress-responsive gene providing cytoprotection to the host and cross-talk with c-Jun N-terminal kinase [32], involved in response to stress but also in T-cell activation and apoptosis, mechanisms of tolerance to infection.

On this background, we hypothesized that iron-induced antioxidant response have a similar impact on malaria to that of hereditary erythrocytopathies, enhancing innate response and preventing tissue damage of toxic products during *Plasmodium* infection. To test it, in the present study we have evaluated the effect of conditioning iron supplementation in a lethal *P. yoelii yoelii* 17XL (*PyL*) model of infection. Iron supplementation in mice was achieved by injecting iron dextran daily during 21 days. On day 19 after treatment starts, a lethal malaria infection was triggered. Mice were investigated for cellular innate responses and redox defence during the early 5 days of infection to observe an early protection against parasitemia and changes in spleen leukocyte populations. In addition, intrinsic iron overload, using an animal model of hemochromatosis, was also studied for *PyL* infection susceptibility with identical progression than wild-type mice.

2. Materials and methods

2.1. Ethics statement

All the experiments were approved by the Committee of Animal Experimentation of the Universidad Complutense de Madrid in agreement with National (R.D. 53/2013) and European (2010/63/CE) legislation.

2.2. Animal groups: infection and iron supplementation

Six-weeks-old female BALB/cAnNHsd mice were purchased from Envigo (Barcelona, Spain), housed under standard conditions and supplied with food (Envigo standard diet containing 50 mg iron/kg) and water *ad libitum*. The rodent malaria parasite *PyL* was kindly provided by Dr. Virgilio Do Rosario (Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa) and stored in liquid nitrogen after serial blood passages in mice.

Sixty mice per experiment were divided in 4 groups (n = 15). Group A (healthy control composed of non-infected animals) and group B (infection control composed of infected animals) included non-iron supplemented mice (Fig. 1). Group C (iron supplemented control composed of non-infected animals) and group D (composed of iron supplemented and infected animals) were treated with iron-dextran 100 mg/kg/day (Sigma, St Louis, MO, USA) for 21 days, since − 19 to day 1 post-infection (p.i.). For infection, mice from groups B and D were intraperitoneally (i.p.) inoculated with 10^6 *PyL*-infected erythrocytes (iRBCs) from previously infected mice in 0.1 mL phosphate buffered saline (PBS) (Fisher BioReagents, Belgium). As standard procedure in this lethal model of malaria, after infection, p-aminobenzoic acid (Merck, Germany) at a final concentration of 0.05% (w/v) was added to the drinking water of all mice to provide to the circulating parasites with sufficient quantities to survive *in vivo* [33]. Parasitemia and parasite forms were monitored daily by microscopy examination of Wright's-stained thin blood smears.

For the studies of lethal malaria infection in hemochromatosis mice, three 5-month female $Hfe^{-/-}$ mice on a C57BL/6J background (strain B6 129P2-Hfetm1gfn /J from The Jackson Laboratory, Bar Harbor, ME, USA) [34] and three 5-month wild-type C57BL/6J (from Envigo) as control were used. The animals were bred under standard conditions and supplied with food and water *ad libitum*. In the study with the $Hfe^{-/-}$ mice and its corresponding controls, the chow was chosen with an iron content of 140 mg/kg, considered normal but containing some increase in comparison with the iron-supplementation study (50 mg/kg), to facilitate iron overload in $Hfe^{-/-}$ mice (not supplemented with iron). Animals were inoculated with 1×10^5 *PyL*-iRBCs from previously infected mice in 0.1 mL PBS. Infection progress was monitored daily by staining blood smears with Wright's eosin methylene blue solution (Merck) followed by iRBC counts under the microscope.

2.3. Anaemia assessment

A drop of blood was taken from each mouse tail on days 1, 3 and 5 p.i. at the same time every day, and haemoglobin (Hb) concentration was determined with the HemoCue Hb 301 analyzer (HemoCue AB, Sweden) and RBCs counted using a Neubauer Chamber. Percentage of reticulocytes with respect RBC and parasite forms where counted under microscope.

2.4. Serum alanine aminotransferase activity

After isoflurane anaesthesia, total blood was taken from each mouse subclavian vein and centrifuged at 1500 × g during 10 min to obtain the serum. The activity of serum alanine aminotransferase (ALT) to

monitor latent liver injury [35] was determined using Spinreact kit (Spinreact, Mexico) adapted to 96-well plates according to the manufacturer's instructions. Enzyme activity was defined in U/L (μmol · $min^{-1} L^{-1}$).

2.5. Non-heme iron in serum

Iron concentration in sera was estimated colorimetrically using a ferrozine-based iron assay kit (Biosystems, Spain) following the manufacturer's instructions. The test is based on the release of Fe^{3+} from transferrin by guanidinium, which is reduced to ferrous iron (Fe^{2+}) by ascorbic acid. Fe^{2+} reacts with ferrozine forming a coloured complex that was measured at 560 nm in a multiwell plate in the Varian Cary 50-Bio spectrophotometer with the plate reader accessory (Agilent Technologies, USA).

2.6. Flow cytometry of spleen cell populations

Five mice of each group were sacrificed on days 1, 3 and 5 of p.i. and spleens were removed. Cells from individual mouse were separately incubated with anti-CD16/32 (clone 93; Thermo Fisher Scientific, eBioscience, USA) and subsequently with anti-CD45R/B220 (RA3-6B2), anti-IgM (II/41), anti-CD4 (GK1.5), anti-CD8 (53–6.7) (BD Pharmingen, San Diego, CA, USA); anti-IgD (11-26c), Mac3 (M3/84), anti-MHCII (M5/114.15.2), anti-CD44 (IM7) (Thermo Fisher Scientific); anti-CD11c (N418) (ABD Serotec) or anti-CD3 (145-2C11) (BioLegend, USA) grouped in three combinations of FITC, PE, PE-Cy5 or APC. Washing steps were made with FACS buffer (PBS with 2% fetal calf serum, FCS). Stained cells were fixed in 2% paraformaldehyde solution and flow cytometric acquisition was performed using a FACSCalibur flow cytometer (BD Biosciences). Data was analysed using FCSExpress software (*De Novo* Software, Los Angeles, CA).

2.7. mRNA expression analysis

To determine mRNA expression of interferon gamma (IFN-γ) in spleen and hepcidin (HAMP), hemoxygenase-1 (HO-1), superoxide dismutase 1 (SOD-1) and catalase (CAT) in liver, we isolated the mRNA on days 1, 3 and 5 p.i. using the GeneJET RNA Purification Kit (Thermo Fisher Scientific) including DNAse I (Thermo Fisher Scientific) digestion according to the manufacturer's instructions. cDNA was synthesized using the RevertAid RT kit (Thermo Fisher Scientific) to subsequently use it for Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) in an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Independent PCR reactions were set for the commercial mixtures of the mouse specific primers and probes corresponding to the gene sequences of *interfg*, *hamp*, *hmox1*, *sod1*, *cat* and the housekeeping β-actin gene (Applied Biosystems assays Mm00801778_m1, Mm04231240_s1, Mm00516007_m1, Mm01700393_g1, Mm00437992_m1 and Mm01205647_g1 respectively). All PCR reactions were set with Maxima Probe/ROX qPCR Master Mix (2 ×) (Thermo Fisher Scientific). PCR reactions included an uracil DNA glycosylase pre-treatment of 2 min at 50 °C, an initial incubation of 10 min at 95 °C for polymerase activation, followed by 40 cycles (melting 15 s at 95 °C, annealing and extension 1 min at 60 °C). Relative changes in gene expression were calculated using the comparative $2^{-\Delta\Delta CT}$ method [36] that directly relates PCR signal of target transcripts in the treated groups (B, C or D) to the untreated control A. Triplicate qRT-PCR experiments were done at least twice as a reproducibility proof.

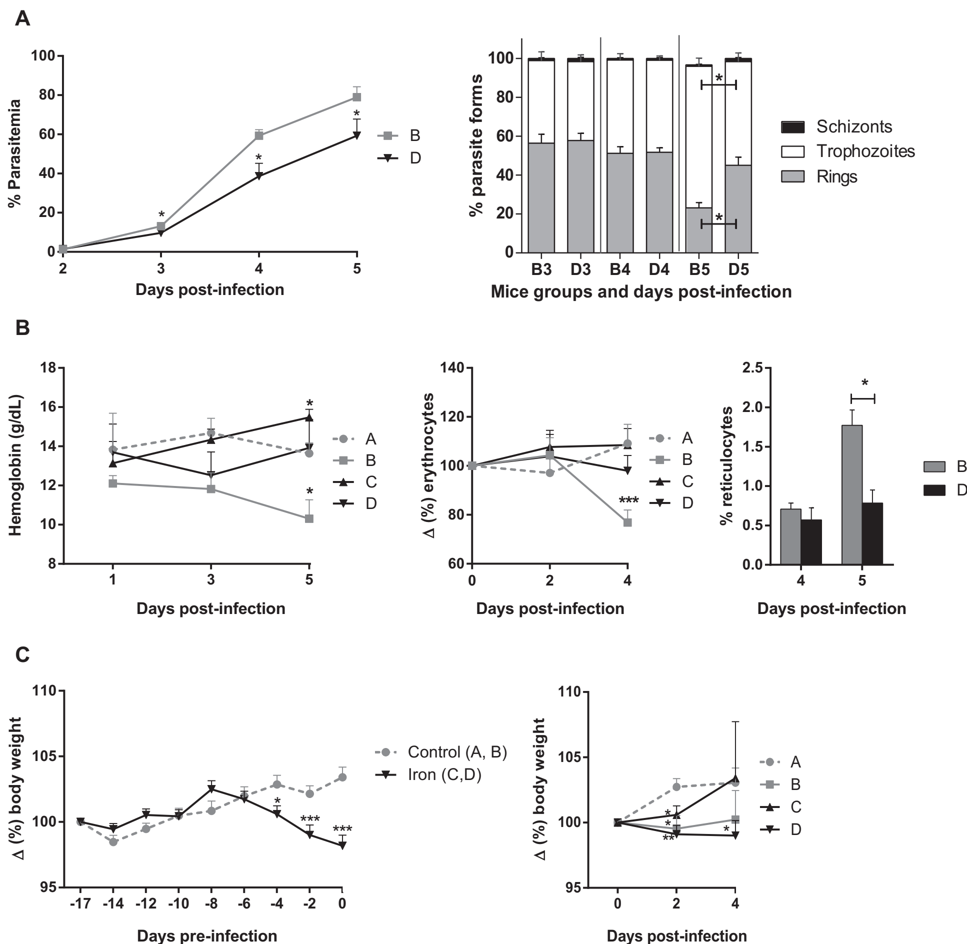

Fig. 2. Progress of infection and anaemia. A) Parasitemia and parasite forms counts in *PyL* infected mice (groups B, D). **B)** Haemoglobin concentration and erythrocyte percentage in comparison to day 0 of all experimental groups; and percentage of reticulocytes in red blood cells counts of the infected groups. **C)** Change of body weight from day −19 to day 0 p.i. and after day 0. ***p < 0.001, **p < 0.01, *p < 0.05 comparing to uninfected untreated mice (group A) when more than one group is shown. Representative graphs show mean ± SEM from 2 to 5 independent experiments (n = 60).

2.8. Statistical analysis

Statistical analysis of quantitative variables was performed using the non-parametric Mann-Whitney test or the parametric Student's *t*-test to compare significant differences between individual groups. To study the linear relationship between continuous variables, Spearman nonparametric correlation coefficients were calculated. All analyses were performed with Prism6 software (GraphPad Software Inc., La Jolla, CA, USA). A $p \leq 0.05$ was considered significant.

3. Results

3.1. Iron supplementation defers parasitemia growth during malaria infection

To examine the influence of iron body levels on the progression of lethal malaria, an experimental infection in mice was performed using four groups: A) control non-infected; B) control infected; C) iron supplemented; and D) iron supplemented and infected (Fig. 1). For iron-supplemented groups (C and D), iron dextran was daily inoculated from day − 19 to 1 p.i. and the infected groups (B and D) were injected at day 0 p.i. with *PyL*-iRBCs.

On group D, the iron supplementation promoted a significant delay in the parasitemia development along the 5 days p.i. follow up with a significant arrest of rings forms by day 5 p.i. (Fig. 2A). In addition, group D eluded the malaria concomitant anaemia (loss of Hb and erythrocytes) and reticulocytosis (Fig. 2B). Nevertheless, it was observed a decrease of body weight the last four days of the treatment (days − 4, − 2 and 0 p.i.) comparing to not supplemented mice (Fig. 2C). The infection had also a detrimental impact on mice weight as both B and D groups kept decreasing weight on days 2 p.i. and/or 4 p.i.

As expected, plasma iron concentration increased in the iron-dextran supplemented mice compared to controls with around 4,5-fold higher levels at day 1 p.i. (Fig. 3A) and, consequently, the liver expression of *hamp*, the gene encoding hepcidin, was induced in them (Fig. 3B). Latent liver injury was monitored through serum ALT activity that significantly increased at days 1 and 3 p.i. in the iron-dextran supplemented groups but which rapidly returned to basal levels at day 5 p.i. (Fig. 3C).

3.2. Macrophages and dendritic cells are strongly expanded in spleen upon iron-supplementation and boosted by infection

The infection, but not the iron supplementation by itself, promoted splenomegaly, increasing both, the organ weight and the total number of cells (Fig. 4), which in turn was directly correlated with the parasitemia kinetics ($r = 0.8$ and $p < 0.001$ in both infected groups B and D).

Moreover, iron-dextran supplementation boosted the spleen cellularity induced by infection at day 3, which concluded with a significantly higher spleen mass by day 5 p.i. Thus, as an essential part of the innate immune response from the spleen, activated dendritic cells (DCs) ($CD11c^+MHCII^+$) and macrophages ($Mac\text{-}3^+MHCII^+$) were examined by flow cytometry (Fig. 4B).

Iron supplemented mice (groups C and D) showed a decreased percentage of spleen DCs by day 1 p.i. Then, when parasitemia showed up in iron supplemented mice (group D), DCs normalized its values, which remained in it until day 5. Contrary, the control infected mice (group B) showed a clear decreased percentage of DCs by day 5 in comparison with uninfected control. Nevertheless, when looking realistically into the total amount of spleen DCs (given the different spleen size at each group) the figures followed a clear expansion as a consequence of infection (× 3 at day 3 or × 7 at day 5, in comparison with uninfected control) which, remarkably, were strongly influenced by iron supplementation (× 9 at day 3 or × 11 at day 5, in comparison with the iron supplemented-uninfected control).

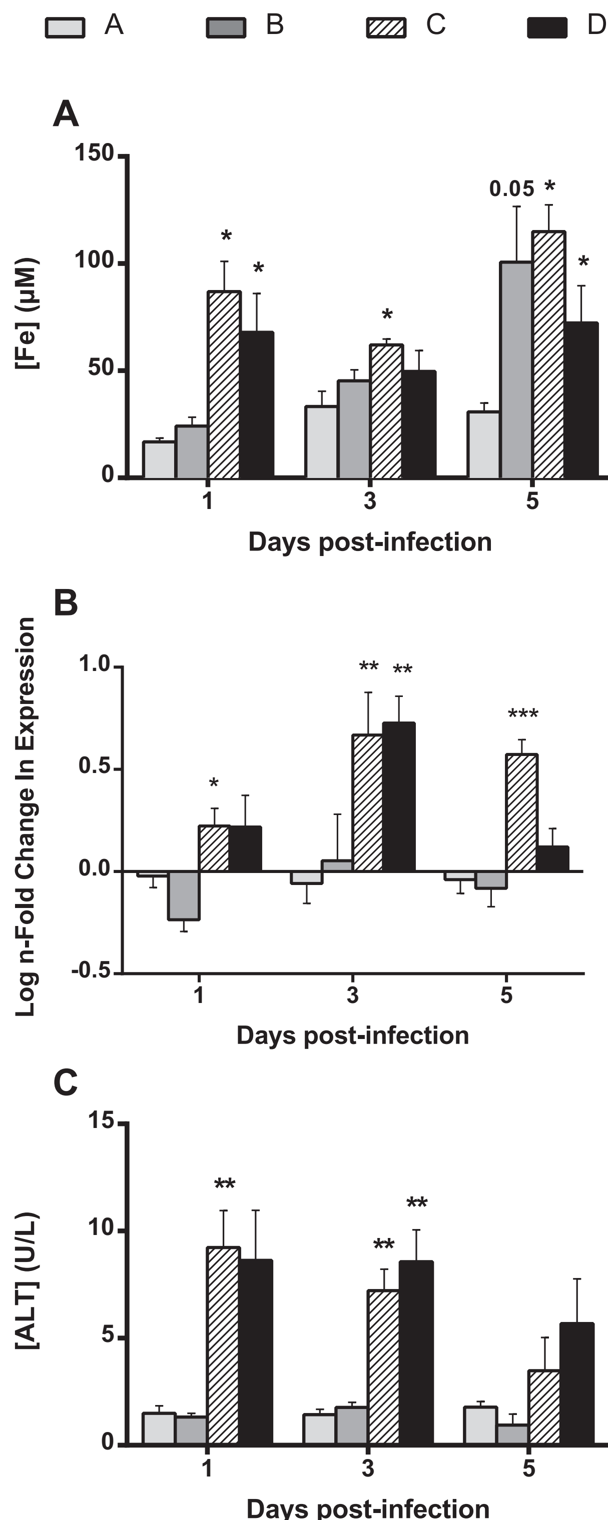

Fig. 3. Markers of iron conditioning. Data from control group (group A), iron supplementation groups (groups C, D) and *PyL* infected (groups B, D). **A)** Iron concentration in sera. **B)** Comparative mRNA expression of hepatic hepcidin calculated by the $2^{-\Delta\Delta CT}$ method (absolute values of transcript copy numbers are given in Supplementary Table S1). **C)** ALT enzyme activity in sera. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ or $p = 0.05$ comparing to uninfected untreated mice (group A). Mean ± SEM of 2 independent experiments with $n = 5$ per time point and group.

Conspicuously between 1 and 5 days, spleen macrophages, independently of the infection status, showed significant increase in both, percentage and total cell amount in the iron supplemented mice with

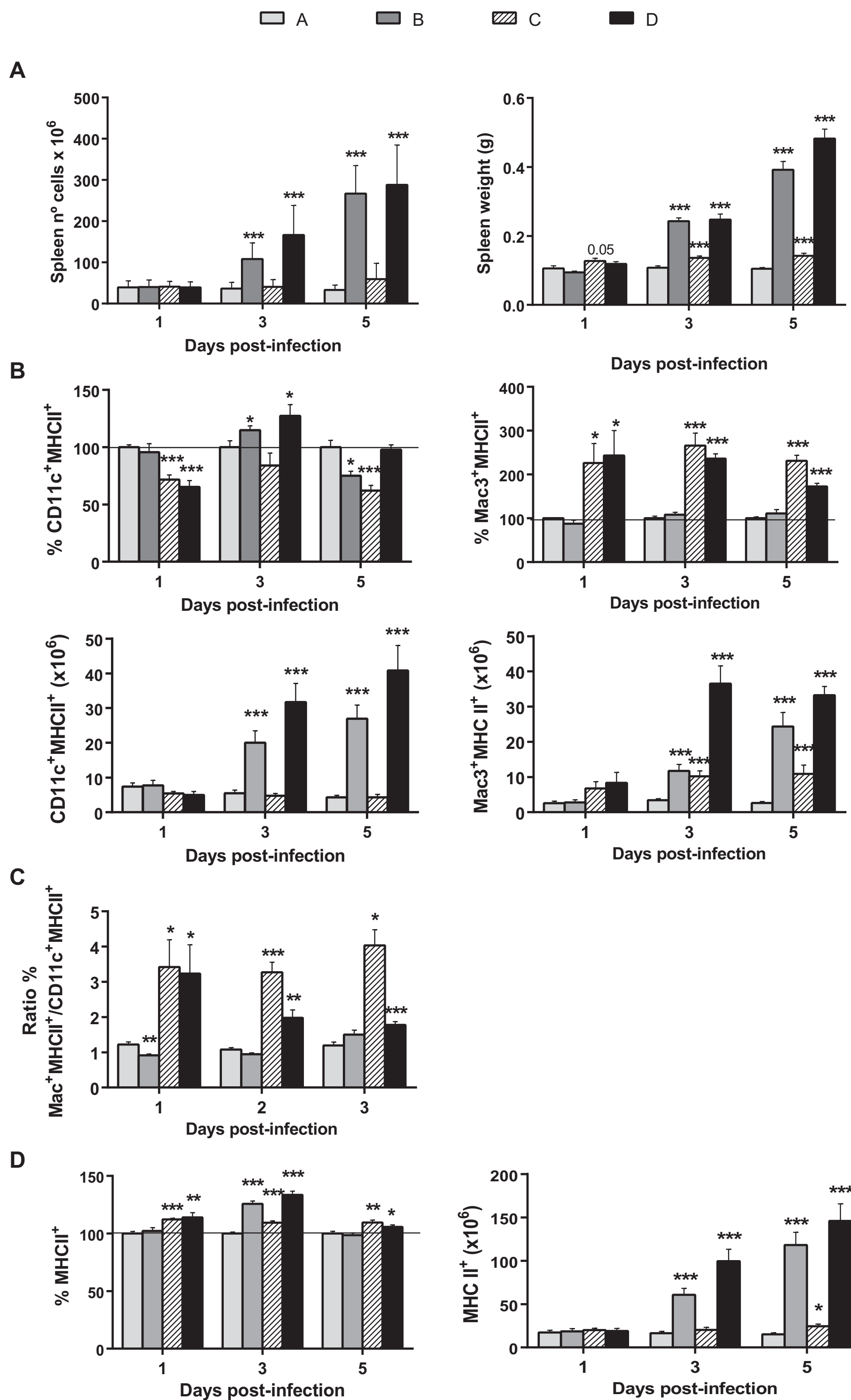

Fig. 4. Flow cytometry analysis of innate immune cells. Spleen cell counts in the four experimental groups (see Fig. 1). **A**) Spleen total cell counts and weight. **B**) Frequency and absolute numbers of macrophages (Mac3$^+$ MHC II$^+$) and DCs (CD11c$^+$ MHC II$^+$). **C**) Macrophage/DC ratio. **D**) Frequency and absolute numbers of antigen presenting cells (MHC II$^+$). Cell percentage given with respect to total leukocytes was normalized to group A mice value. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ with respect to group A. Mean ± SEM of 2 independent experiments, each with n = 5 per experimental point and group.

respect to its respective controls. Differently to DCs, at day 1 p.i. macrophages already showed between × 3 and × 4 increased numbers in iron overloaded mice. Furthermore, infection also boosted macrophages and consequently, absolute numbers of spleen macrophages notably expanded in groups B and D. Thus, iron supplementation in mouse enhanced the innate cell response in spleen upon infection, in parallel with a slower growth of blood parasites.

Besides, as seen in Fig. 4C, the ratio between macrophages and DCs was always > 1 in iron overloaded mice. Among the non-infected mice, iron-overloaded group C was permanently three times higher than control. The infection promotes progressively diminished macrophage/DC ratios that decreased slower in the iron-overloaded group D in comparison with group B.

To assess the antigen presentation capacity during the infection, we examined the overall presence of leukocytes expressing MHC II molecules (Fig. 4D). Iron-supplemented mice (groups C and D), as earlier as day 1 p.i., showed in the spleen higher percentages of MHC-II expressing cells than controls. Infected mice increased the percentage of this

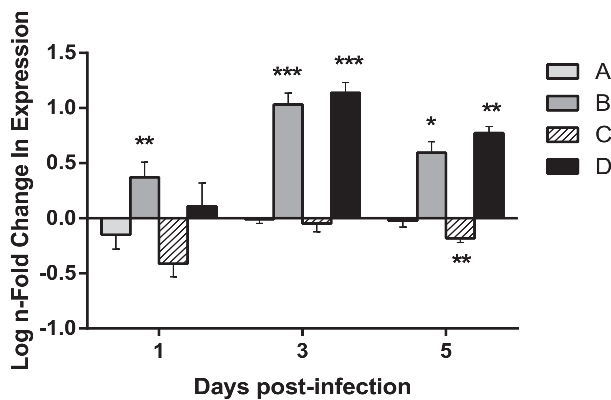

Fig. 5. Expression of IFN-γ. Comparative mRNA expression analysis of IFN-γ in spleen calculated by the $2^{-\Delta\Delta CT}$ method (absolute values of transcript copy numbers are given in Supplementary Table S2). Group A, control healthy mice; group B, infected mice; group C, iron inoculated mice; and group D. iron-treated and infected mice. ***p < 0.001, **p < 0.01, *p < 0.05 with respect to group A in identical conditions. Data shows the mean ± SEM of 2 independent experiments, each with n = 5 per experimental point and group.

cell population at day 3 and it was only maintained at day 5 in the iron-supplemented mice. Similar to total spleen cell kinetics, the number of cells MHC II$^+$ dramatically increased in infected mice (B and D groups) reaching numbers × 8–10 higher than healthy controls.

We determined the production of IFN-γ mRNA in spleen as an indicator of inflammation in iron supplemented mice. We observed that mRNA expression of IFN-γ was induced by *PyL* infection with a minor interaction by day 5 with iron supplementation (Fig. 5).

3.3. Iron-supplementation modify splenic B cell maturation ratios

Since *Plasmodium* primary infection generates a very large population of splenic B cells (B220$^+$), this compartment and its differentiation to immature transitional 1 (T1) and mature B cells was investigated for changes caused by the conditioning iron treatment. As shown in Fig. 6A, the iron treatment groups (C and D) opened from day 1 p.i. with higher percentage of splenic B cells (B220$^+$) than their respective control groups but the difference is abrogated in comparison with the B cell growth caused by infection and splenomegaly as parasitemia showed up by days 3 and 5 p.i.

To further explore the maturation of B cells following infection, the expression of surface IgM and IgD was determined in B220$^+$ cells (Fig. 6B). The infection (groups B and D), and mainly by day 5 at maximum parasitemia, promoted a significant increment of the relative percentage of switched B cells (IgM$^-$ IgD$^-$) in detriment of mature and immature B cells, indicating their differentiation to antibody-producing cells. The iron treatment by itself (group C), did not change the frequencies of B cell subpopulations comparing to control mice. Following the observed splenomegaly, the absolute numbers of total B cells and each B cell subpopulations increased in infected mice with no influence by iron treatment (Fig. 6C). The calculation of ratio between subpopulations rendered at day 5 p.i. a clear increment of switched B cells in infected mice with respect mature and T1 B cells, while the latter maintained the relation M/T1 at constant values in all mice (Fig. 6D).

3.4. T cell kinetics response to malaria infection and activation to iron supplementation

Two populations of T cells were considered in the spleen. The CD4 T cells (CD4$^+$ CD3$^+$) which play a role in fighting blood-stage malaria, and CD8 T cells (CD8$^+$ CD3$^+$) that mainly participate in the defence against liver-stage malaria [37]. The percentage of CD4 T cells in the spleen diminished by iron overload at day 1 p.i. and further decreased by the infection as observed at days 3 and 5 p.i. (Fig. 7A left panel). Nevertheless, the total number of CD4 T cells increased significantly only in infected mice, with increased values caused by iron treatment (Fig. 7B left panel), in line with the increase of spleen cellularity. Frequency of CD8 T cells remained steady although with some decline just after treatment and at day of maximum parasitemia, but again, the total number of them increased at days 3 and 5 p.i. (Fig. 7A and B, right panel). Iron treatment did not change the absolute number of none of the T cell populations in comparison to the respective controls.

To further study T lymphocytes function, we measured the expression of CD44, a ligand for hyaluronic acid, which is up-regulated in activated cells mediating rolling and adhesion during the migration of activated lymphocytes to sites of immunity [38]. Within the CD4 T cell population (CD4$^+$ CD3$^+$), the percentages of spleen activated cells (CD44$^+$) only increased in the infected groups from day 3 p.i. onwards with a maximum of around 40% (Fig. 7C, left panel). On the contrary, CD8 T cells only showed a minimal and transient increase of activation of about 3–4% in the infected groups (Fig. 7C, right panel). The ratio between numbers of CD4 T cells and their main antigen presenting cells, the DCs, in the spleen increased by iron treatment, but decreased as the infection progressed (Fig. 7D).

3.5. Altered expression of redox enzymes caused by infection and iron supplementation

According to the potential effect of iron in promoting oxidative reactions, we analysed in the liver the expression of the major effector genes participating in the redox defence: *hmox1* encoding the heme catabolizing enzyme HO-1, *sod1* that encodes the enzyme degrading superoxide radical into hydrogen peroxide and oxygen, and *cat*, encoding the hydrogen peroxide converting enzyme into water and oxygen. Hence, in the iron supplemented groups (C and D), iron up-regulated the *hmox1* expression from day 1 p.i., while *sod1* and *cat* followed the opposite trend of early downregulation (Fig. 8). On the other hand, the infection by itself caused a remarkable boost in the expression of *hmox1*, reaching the maximum in both infected groups by day 5 p.i., and a downregulation of *sod1* and *cat*. The effect of iron on *sod1* and *cat* expressions was then notably exacerbated by the infection reaching the maximum reductions on group D, and exceeding group B.

3.6. Intrinsic iron overload in hemochromatic mice Hfe$^{-/-}$ does not protect from lethal malaria

To analyse the infection in a model of natural iron overload we injected 1×10^5 *PyL*-iRBCs in hemochromatic Hfe$^{-/-}$ mice on a C57BL/6J background. The parasitemia, survival and weight of Hfe$^{-/-}$ mice followed the same kinetics than in wild-type mice, dying by day 7 p.i. with very high parasitemia rates (Fig. 9). This is a marked difference with the extrinsic iron overload by iron dextran showed above. Consequently, we did not analyse in detail the immune response in these experiments.

4. Discussion

The use of iron supplementation in mass programs to prevent anaemia is controversial due to the potential presence of undiagnosed infective pathogens that can uptake iron as a micronutrient. When human body face iron overload, since no iron excretory pathways exist, cellular homeostatic mechanisms must balance between potential redox benefits and negative effects as toxicity and microbial iron acquisition [39]. To study the iron overload effects in redox and immune responses during an infection we analysed here the iron supplementation on an early response to a lethal *PyL* malaria challenge in BALB/c mice, given that malaria and anaemia share the same high-risk groups in populations of regions where both diseases are co-endemic [40]. In addition, malaria is by itself a cause of anaemia [40]. For this purpose, we generated an iron-overloaded mouse model, by the inoculation of iron-

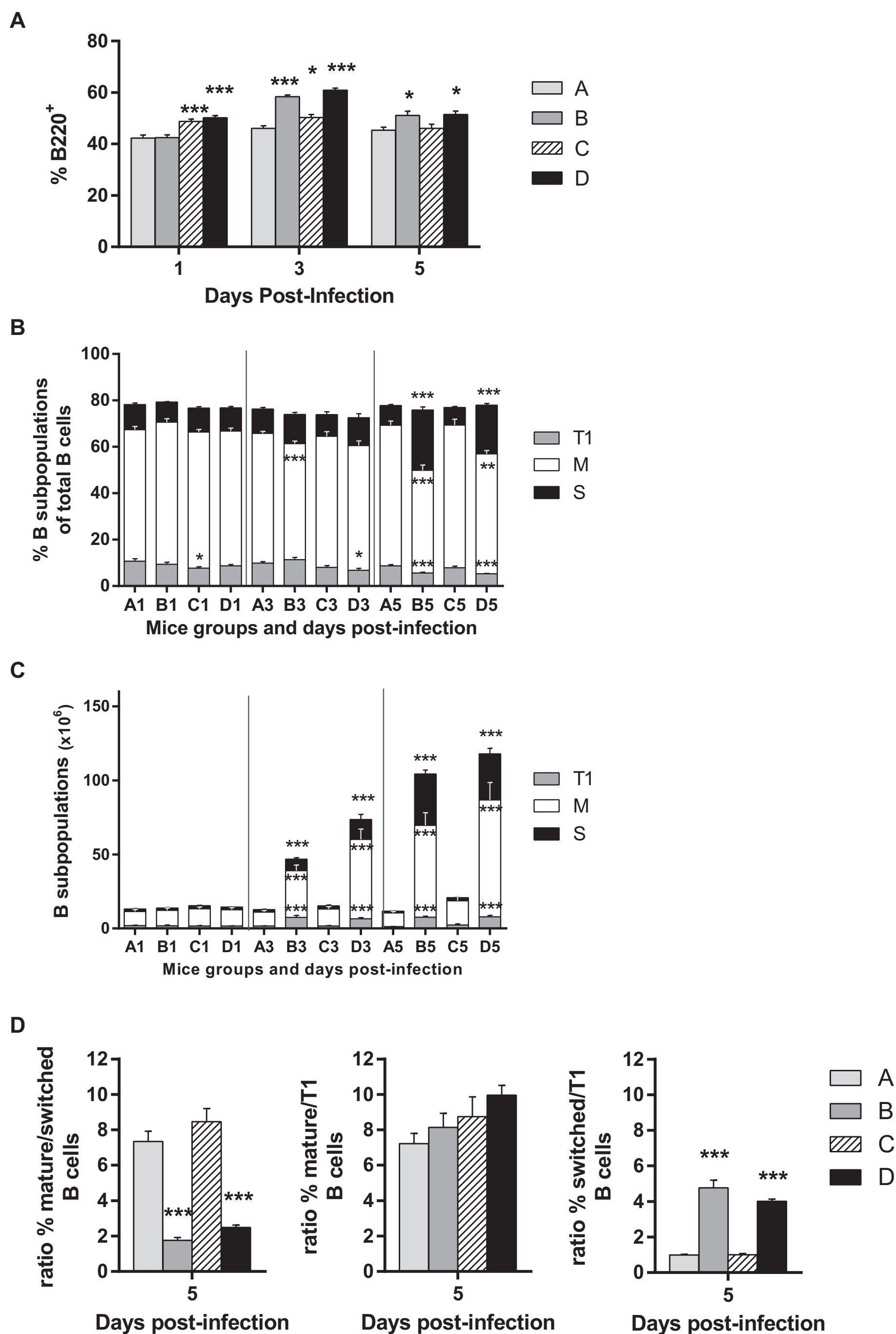

Fig. 6. Spleen B lymphocytes maturation. Spleen cells from the four experimental mice groups (see Fig. 1) were labelled to analyse B cells ($B220^+$) by flow cytometry. **A**) Percentage of B cells with respect to total leukocytes. **B**) Percentage, and **C**) absolute number of B cell subpopulations: transitional 1 (T1) ($IgM^{hi}IgD^{low}$), mature (M) ($IgM^{low}IgD^{int}$) and class-switched (S) (IgM^-IgD^-). **D**) Ratios between B cell subpopulations at day 5 p.i. Data shown is the mean value ± SEM of two independent experiments, each with n = 5 per time point. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ with respect to the healthy group A.

dextran 100 mg/Kg during 21 days at a total dose of 42 mg/mouse, which presented an iron concentration in serum increased 4–5 fold. Similar total doses in rats have been also reported to increase 2-fold serum iron [41]. As we could expect from a physiological response to iron overload and inflammation [42,43], the expression of hepatic hepcidin was upregulated. Hepcidin modulates blood iron concentration by binding to the iron exporter ferroportin, expressed on the cell surface of macrophages and enterocytes, and inducing its degradation [8,44]. Hepcidin, consequently decreases both iron absorption in intestine and iron recycling from macrophages [8] to restore iron levels. In our mice, the iron supplementation also promoted some negative side effects, as a red skin, weight loss and a transitory liver damage, probably caused by lipid peroxidation *via* hydroxyl free radical production [45].

During malaria infection, the rupture of erythrocytes by the parasites results in an accumulation of large amounts of free heme that largely promotes inflammation [46] and oxidation [42]. Here, we tested the hypothesis that a previous exposure to an pro-oxidative agent such iron could activate an anti-oxidative response [32,47] that would fight against oxidative damage in tissues, ameliorating the disease outcome. This mechanism of protection is not new and could be also promoted by intrinsic iron conditioning. The higher rate of heme released from HbS, that significantly increases auto-oxidation rate compared to normal HbA [48], early induces the expression of HO-1 protecting against the cytotoxic effects of free heme [30,49,50]. Thus, individuals carrying the heterozygous HbS allele seem to be better equipped to deal with malaria oxidative stress relying on the HO-1 expression [30,49,51].

In our challenge with the lethal *Plasmodium* strain we observed that the iron supplementation delayed parasitemia during the first days of

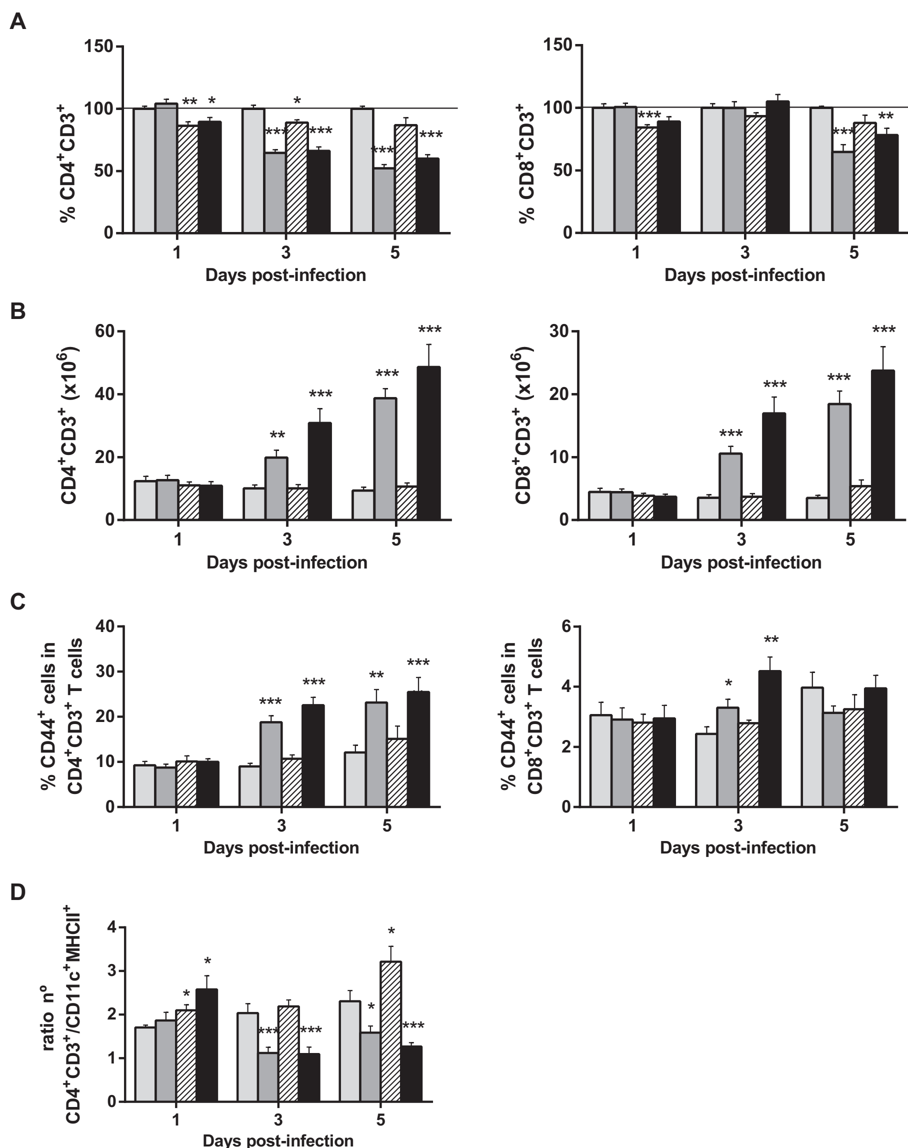

Fig. 7. Spleen CD4 and CD8 T cells kinetics. Splenocytes from the four experimental mice groups (see Fig. 1) were labelled to analyse: **A**) CD4 ($CD3^+CD4^+$) and CD8 ($CD3^+CD8^+$) T cell percentages with respect to total leukocytes (normalized to group A mice data); and **B**) absolute numbers of CD4 and CD8 T cells. **C**) Percentage of CD44 expressing cells in CD4 (left) and CD8 T (right) populations. **D**) Ratio between numbers of CD4 T cells and DCs. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ with respect to group A. Mean ± SEM of 2 independent experiments, each with n = 5 per experimental point and group.

infection, improving haemoglobin concentrations and decreasing number of reticulocytes, similarly to the iron supplementation in humans that also reduces anaemia [3,18]. This effect is in agreement with the late rings forms of *P. yoelii* seen in the iron-supplemented mice. Not heme, free-iron also induced hepatic *hmox* in our mice before infection, suggesting that any early expression of *hmox* could prepare tissues against the damage caused by the released free heme during malaria infection and inflammation [32,47,52–54], being in fact, a mechanism of host defence against malaria by other *Plasmodium* species in mice [31,32].

The understanding of the interaction between iron body levels and susceptibility to infection in malaria endemic areas is still a challenge [55,56]. Similar to our results in mice, some studies show that morbidity is higher in iron deficient children and reduced by iron supplementation [57,58]. Comparable to humans [18–21], experimentally, iron overload in rats do not increase susceptibility to infections by *P. berghei* [16,17]. On the contrary, other studies support that human malaria could be exacerbated by iron supplementation [5,13,14]. In the same trend, treatment of asymptomatic malaria with iron chelation decrease parasitemia [59]; children with iron deficiency has lower incidence of malaria [15]; and hypoferraemic mice infected with *P. berghei* show a substantial reduction of mortality [60]. It should be noted that the increased amount of free-iron available in our mice did not accelerate parasite multiplication, in contrast with the rationale by which some studies suggest the advantages of using iron chelation to treat malaria [56]. Even, when our daily iron dose in mice (100 mg/Kg) was higher than the recommended in humans [3,11] we did not observe any aggravation of the infection, but on the contrary, a protection. The lack of any protection against malaria in mice with mutated Hfe gene could be explained by a lower increase of serum iron concentration in $Hfe^{-/-}$, less than twice the iron of wild type mice [34] compared to our 4–5 fold-increase, which constitute a wicker oxidative stimulus than the obtained in our iron-supplemented mice. Probably, intrinsic lower doses of iron overload are insufficient to boost the dominant antioxidant defences like *hmox* since $Hfe^{-/-}$ mice do not increase its expression [61].

Since SOD-1 and catalase provide a key detox against superoxide radicals from malaria infection, the first converting free radicals into H_2O_2 and the second removing it [62]. We analysed their expressions in our experimental lethal challenge. Both transcriptions were downregulated first by iron overload and later by the infection. SOD-1 and catalase downregulation in brain is associated with experimental cerebral malaria progression in mice infected with *P. berghei* [63]. However, their behaviours in humans are not always homogeneous, since SOD-1 is a biomarker of severity in *P. falciparum* or *P. vivax* infection in humans [62,64], but catalase shows diminished enzyme activity in

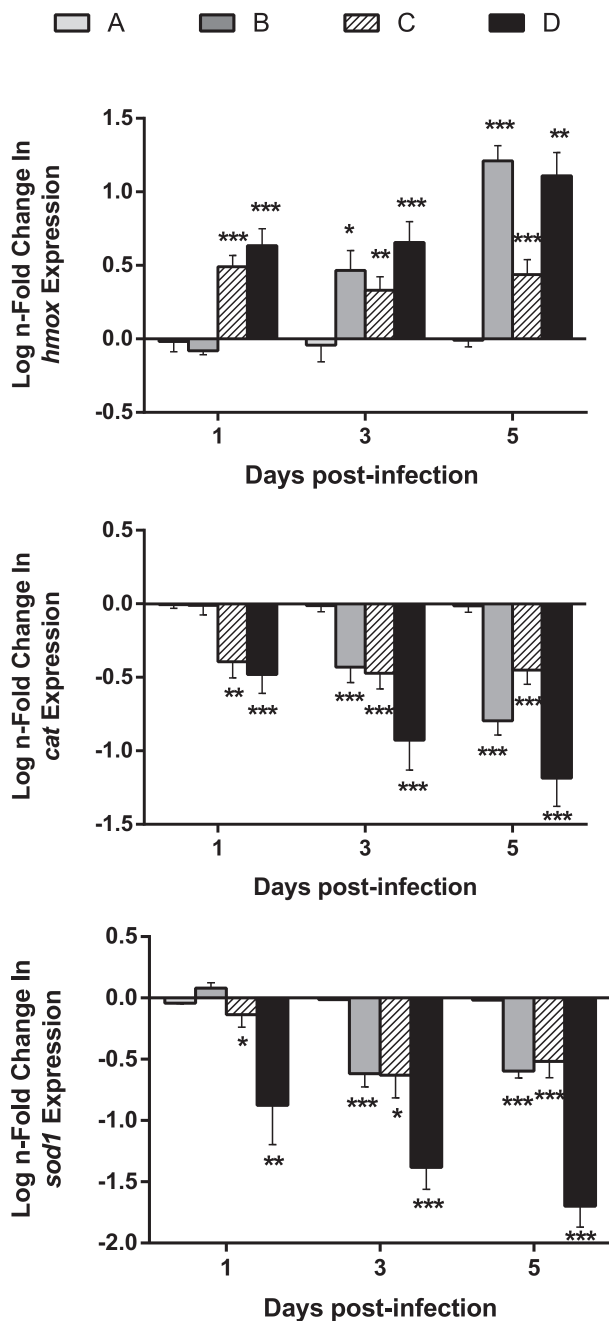

Fig. 8. Expression of anti-oxidant enzymes. Comparative hepatic mRNA expression analysis of *hmox1* (upper panel), *cat* (middle panel) and *sod1* (lower panel) between control healthy mice (group A), and infected mice (group B), iron-treated mice (group C) and iron-treated and infected mice (group D). Data shown was calculated by the $2^{-\Delta\Delta CT}$ method (absolute values of transcript copy numbers are given in Supplementary Table S3). Mean ± SEM of 2 independent experiments, each with n = 5 per experimental point and group. ***p < 0.001, **p < 0.01, *p < 0.05 with respect to group A.

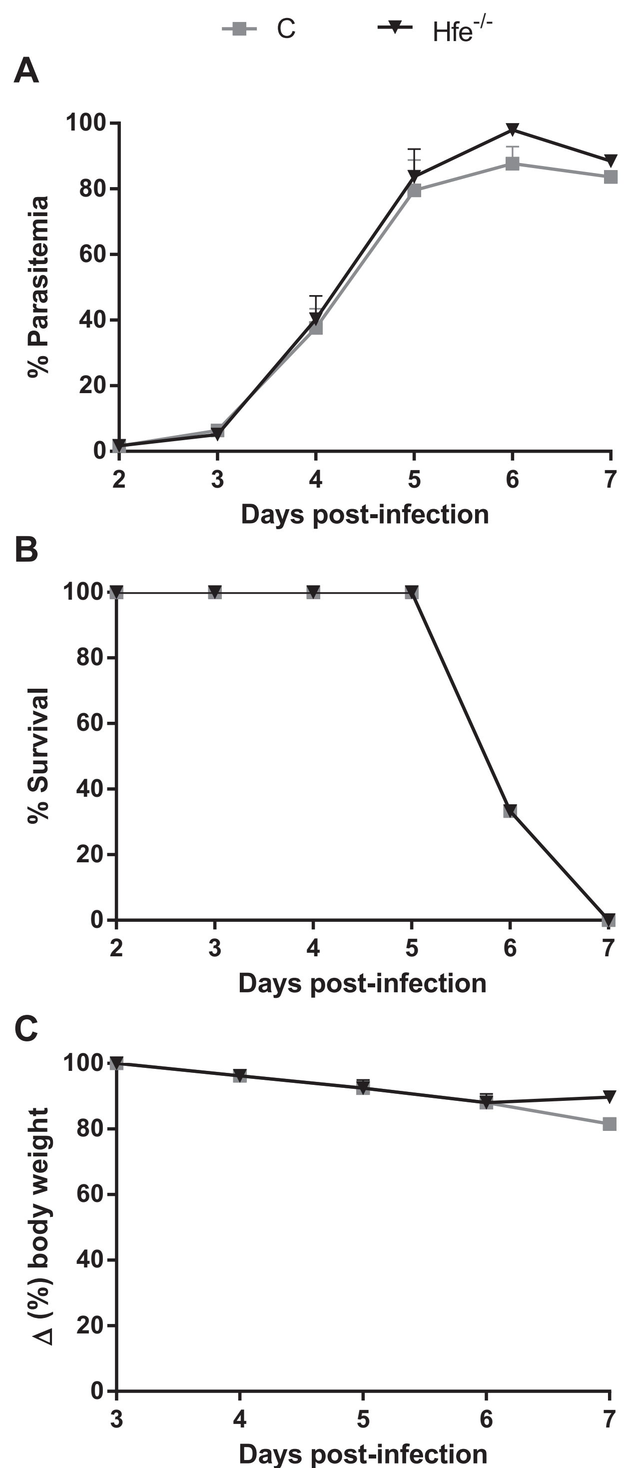

Fig. 9. *PyL* malaria infection in hemochromatosis Hfe$^{-/-}$ mice. A) Parasitemia; **B**) survival and **C**) body weight change after *PyL* infection in C57BL/6J Hfe$^{-/-}$ and wild type mice (C). Representative graphs show mean ± SEM of one experiment with n = 3 per group.

serum of infected patients [62]. Whether switching of these enzymes by different parasite species and/or tissues could regulate infection development remains to be investigated.

There is an intricate relationship between host iron metabolism and the innate immune system [4]. To understand how iron modulated the innate immune response, we analysed the phenotype of the immune cell populations at days 1, 3 and 5 p.i. in the spleen, a lymphoid organ with a critical role in the defences against malaria [65]. In our mice, independently of iron supplementation, a splenomegaly was developed after the infection, which is a major sign of malaria. With respect innate cells, iron supplementation decreased DCs and dramatically increased macrophages percentages at the end of the treatment in comparison with control mice. Thus, infected iron-overloaded mice started the first day of infection with 3-fold more macrophages than DCs while control infected mice had nearly equal amounts of both of them. Innate immune regulation requires a close interplay of monocytes with their macrophage and DC progeny [66]. Not much research has addressed the interaction between iron loading and DCs function [67], and we have observed a preference of monocyte recruitment into spleens to

differentiate into macrophages. Consequently, macrophages were the only immune population whose absolute numbers increased in the spleen of non-infected iron overloaded mice, remaining the DCs, T CD4, T CD8 and B cells in normal numbers. This adaptation fits with the central role of macrophages activating inflammation and maintaining iron homeostasis, recycling iron from senescent RBCs to make it available for redistribution to other tissues *via* transferrin [42,44].

On the other hand, DCs, macrophages and B cells are capable of presenting antigens to T CD4 cells *via* MHC class II what is absolutely required for the resolution of infections caused by *P. yoelii* [68]. The iron supplementation induced these antigen-presenting cells during the whole experiment while the infection alone was only transiently linked to the frequency increase of these cells. Since iron supplementation expanded earlier splenic macrophages and antigen presenting cells, we further examined in spleen the acquired immune cells. The number of DCs stimulating CD4 T cells is determinant in the T cells outcome, being a low CD4/DCs ratio inhibitory to T cell proliferation *in vitro* [69]. This ratio was in fact nearly halved in infected mice, but increased in iron overload mice before infection, what could have influenced the adaptive response from the very beginning of the infection. Regarding T lymphocytes, their percentage in iron-supplemented uninfected mice remained unchanged. The infection, independently of the iron status, activated T $CD4^+CD44^+$ and B cells (IgM^-IgD^-) in all mice at the time that parasitemia showed up, with a concomitant increase in the pro-inflammatory IFN-γ mRNA levels. On the contrary, T CD8 cells remained mostly unchanged in their percentage and activation state, in agreement with their known non-protective role against blood stage *P. yoelii* infection [70]. On the other hand, while iron treatment promoted the proliferation (without interfering in the maturation) of splenic B cells with respect total splenocytes by the end of the treatment period (day 1 pi), the infection alone did not stimulate it until day 3 pi. This condition could confer an early advantage in controlling the parasitemia in the iron-overloaded mice, since B cells have a key role in controlling *P. yoelii* malaria infections [71].

5. Conclusions

Here we show that iron supplementation, through mechanisms related with antioxidant and cellular immune responses, was capable of delaying a lethal malaria infection. A fine tuning between innate cell activation and oxidative-stress conditioning to push acquired immunity seems to be interplayed by iron. Future studies focused to modulate these early innate responses with respect survival are needed in this malaria model, since the understanding of such responsive equilibrium in humans should be considered in the risk-benefit assessment of iron supplementation in malaria endemic areas.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgements

This work was funded by grants from Fundación Ramón Areces and MINECO projects BIO2013-44565R and BIO2016-77430R.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2017.09.027.

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