

Mycobacterium avium Infection Induces H-Ferritin Expression in Mouse Primary Macrophages by Activating Toll-Like Receptor 2

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Abstract

Important for both host and pathogen survivals, iron is a key factor in determining the outcome of an infectious process. Iron with-holding, including sequestration inside tissue macrophages, is considered an important strategy to fight infection. However, for intra-macrophagic pathogens, such as *Mycobacterium avium*, host defence may depend on intracellular iron sequestration mechanisms. Ferritin, the major intracellular iron storage protein, plays a critical role in this process. In the current study, we studied ferritin expression in mouse bone marrow-derived macrophages upon infection with *M. avium*. We found that H-ferritin is selectively increased in infected macrophages, through an up-regulation of gene transcription. This increase was mediated by the engagement of Toll like receptor-2, and was independent of TNF-alpha or nitric oxide production. The formation of H-rich ferritin proteins and the consequent iron sequestration may be an important part of the panoply of antimicrobial mechanisms of macrophages.

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Introduction

As an essential nutrient for both host and invader, iron plays a central role in determining the outcome of infections. Infection leads to a re-distribution of iron in the body of vertebrate animals, so that less iron is found in circulation and more iron is sequestered inside macrophages [1]. While the decrease in circulating iron is a critical step for the control of the growth of extra-cellular microbes, it is less clear how the increased sequestration of iron inside macrophages impacts on intra-macrophagic infections. There are several microbes that reside and proliferate inside the macrophages of the animals they infect. These agents, including mycobacteria, leishmania, and salmonella, tend to cause chronic infections. Previous work by ours and other groups has shown that iron availability can clearly affect the growth of mycobacteria during experimental infection of mice. In iron-overloaded mice, mycobacteria grow more extensively, while they grow less in mice rendered iron deficient [2–5]. These results can be reproduced *in cellulo* using the model of infection of bone marrow-derived mouse

macrophages, infected with *Mycobacterium avium* [2]. Given the clear evidence that iron availability affects the growth of *M. avium* inside macrophages, it is relevant to understand whether macrophages are able to modulate intracellular iron availability to the microbes they harbour.

Ferritin is a key regulator of the intracellular iron metabolism through the storage of iron. In mammals, cytosolic ferritin is formed by 24 subunits of heavy (H) and light (L) chains, that spontaneously assemble into a shell-like structure, capable of storing up to 4500 iron atoms in its mineral core [6]. H-ferritin has a ferroxidase centre that promotes the conversion of Fe²⁺ to Fe³⁺, and L-ferritin facilitates nucleation and mineralization of the iron core [7]. Ferritin subunits are not interchangeable, as shown by the embryonic lethality of H-ferritin gene deletion [8]. H- and L-subunits are encoded by separate genes, which are differentially regulated [9]. As many other iron-related proteins, ferritin expression can be regulated post-transcriptionally by iron regulatory proteins (IRP), which interact with the *cis*-regulatory iron responsive elements (IRE) present in the 5' UTR of both ferritin mRNAs [10].

In the present work, we investigated the regulation of ferritin in mouse bone marrow-derived macrophages infected with *M. avium*. We show that *M. avium* induces an increase of H-ferritin mRNA, and concomitant protein level, which is dependent on the engagement of Toll-like receptor-2, and independent of the production of TNF-alpha and endogenous nitric oxide.

Materials and Methods

Animals

C57BL/6 mice were bred at IBMC. TLR2-deficient mice, on a C57BL/6 background [11] were bred at the IBMC from a breeding pair kindly provided by Dr Shizuo Akira. TNF-alpha-deficient mice, on a C57BL/6 background, were bred at the IBMC from breeders purchased from B&K Universal (East Yorkshire, United Kingdom). Inducible nitric oxide synthase (iNOS) – deficient mice [12], on a C57BL/6 background, were bred at the IBMC from a breeding pair kindly provided by Drs J. Mudgett, J.D. MacMicking and C. Nathan (Cornell University, New York). All mice were kept at the IBMC animal facility in high efficiency particulate air (HEPA)-filter-bearing cages and fed sterile chow and autoclaved water.

All animal maintenance and manipulations were conducted according to the rules of the IBMC animal ethics committee. This study was carried out in strict accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and 86/609/EEC Directive and Portuguese rules (DL 129/92). The animal experimental protocol was approved by the competent national authority Direcção Geral de Veterinária (DGV) (Protocol Permit Number: 0420/000/000/2011)

Bacteria

Mycobacterium avium strain 2447, smooth transparent (SmT), was isolated from an AIDS patient and given to us by Dr. F. Portaels, Institute of Tropical Medicine, Antwerp, Belgium. The bacteria were grown in Middlebrook 7H9 Broth (Difco) with 0.05% Tween 80 (Sigma). Cultures were harvested during exponential phase, centrifuged, washed in saline with Tween 80, briefly sonicated and stored in aliquots at -80 °C until used.

Macrophage culture

Bone marrow cells were flushed from mice femurs with ice cold Hank's Balanced Salt Solution (HBSS, Gibco), collected by centrifugation and resuspended in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) containing 10% Foetal Bovine Serum (FBS, Gibco) and 10% L929 Cell Conditioned Medium (LCCM), as a source of Macrophage-Colony Stimulating Factor (M-CSF). The cells were distributed in 24-well plates and incubated at 37 °C in a 7% CO₂ atmosphere. Three days after seeding, another 0.1 ml LCCM was added. On the 7th day, the medium was renewed. On the 10th day of culture, when cells were completely differentiated into macrophages, some wells were infected with *M. avium*, by substituting the medium with 0.2 ml of DMEM containing 10⁶ CFU of *M. avium*

(approximately 10 bacteria per macrophage). In uninfected controls, the medium was replaced with an equal volume of DMEM. Cells were incubated for 4h at 37°C in a CO₂ atmosphere and then washed with warm HBSS to remove non-internalized bacteria and reincubated in DMEM, with 10% FBS and 10% LCCM. To block the transcription of the cell, some macrophages were incubated for 15 min with 1 µg/ml Actinomycin D (Sigma) in DMEM at 37 °C before infection. In some experiments, macrophages were incubated with 1 ng/ml of the synthetic diacylated lipoprotein FSL-1 (InvivoGen) in complete medium.

Quantification of H- and L-Ferritin

Macrophages were washed with cold saline and lysed with 400 µl of a solution containing 50 mM Hepes (Gibco), 1% IGEPAL C-630 (Sigma) and 1% proteases inhibitor cocktail P840 (Sigma). Ferritin concentrations in the lysates were determined by ELISA assays using polyclonal antibodies (Abs) raised against mouse recombinant H- and L-ferritin subunits and calibrated with the corresponding recombinant homopolymers. The specificity and the absence of cross-reactivity of the Abs have been previously described [13]. Additional tests have been performed to confirm the absence of cross-reactivity with mycobacterial antigens. The results are expressed as ng of ferritin per mg of total protein in the cell lysate. Total protein content was measured using the BCA protein assay kit (Pierce).

⁵⁵Fe uptake and incorporation into ferritin

BMM uninfected or infected with *M. avium* for 24h were incubated with 2.5 µM (⁵⁵Fe) ferric ammonium citrate (Perkin Elmer) for 6h, at 37 °C in a 7% CO₂ atmosphere. Afterwards, cells were washed with cold saline and lysed with 25 mM Tris-HCl buffer pH 7.4 (Sigma), containing 0.5% Triton X-100 (Sigma) and 1% proteases inhibitor cocktail P840 (Sigma). Total protein content was measured on soluble homogenates using Bio-Rad DC™ Protein Assay (Bio-Rad) and 18 µg were loaded on a native PAGE (7.5% acrylamide, 1.5 mm thick). The gel was dried, subjected to phosphor imaging (Typhoon 8600; Molecular Diagnostics, Amersham Biosciences), and analyzed using the ImageQuant program version 5.1.

Gene expression

Total RNA was extracted using the Micro-to-Midi Total RNA Purification System (Invitrogen) according to the manufacturer's specifications. 2 µg of total RNA was transcribed into cDNA, by a Moloney Murine Leukemia Virus Reverse Transcriptase (Fermentas), using an oligo(dT)₁₈ primer. The primers used for amplification of cDNA were as follows: *Hprt1* (housekeeping) 5'- gtaatgatcagctcaacgggggac -3' (forward) and 5'-ccagcaagcttgcaaccttaacca-3' (reverse); *Fth1* 5'- ggagttgtatgcctcctac -3' (forward) and 5'- gagatattctgcatgcc -3' (reverse). The primers were shown not to co-amplify genomic DNA. All reactions were performed in a total reaction volume of 20 µL with iQ™ SYBR® Green Supermix (Bio-Rad) and carried out in the iQ™5 instrument (Bio-Rad). Baseline thresholds were calculated by Bio-Rad iQ5 program and the threshold cycles (Ct) were used in the REST software [14],

where CT values for target gene were normalized to expression levels of *Hprt1*. Values are reported as n-fold difference relative to the control samples.

Electromobility Shift Assay

BMM were washed with cold PBS and incubated for 10 min on ice with digitonin (Sigma) at 0.007% in sucrose 0.25M and Hepes 0.1M, pH 7.4, and centrifuged 10 min at 1800 g. The supernatant was centrifuged for 1h at 100 000 g and the mitochondria-free cytosolic extract was collected. The samples were kept at -80 °C until use. RNA-protein interactions were performed as described previously [15,16] using 4 µg of cytosolic extract and a molar excess of [alpha-³³P]UTP- labeled H-ferritin IRE *in vitro* transcribed from plasmid pSPT-fer (kindly provided by Dr. L.C. Kühn, ISREC, Switzerland). IRE-IRP complexes were resolved in 6% nondenaturing polyacrylamide gel. Samples were treated in parallel with 2% 2-mercaptoethanol prior to the addition of the ³³P-labeled IRE probe to fully activate IRP-IRE binding activity.

Statistical analysis

Data was analysed using a two-tailed unpaired student's t test.

Results

1. *Mycobacterium avium* infection increases ferritin heavy chain levels in bone marrow-derived macrophages through transcriptional activation

To test whether *M. avium* infection alters intracellular ferritin levels, we infected mouse bone marrow-derived macrophages (BMM) and analysed the ferritin protein levels for up to 5 days. The infection led to a 5.3±0.9 fold increase of ferritin heavy chain in the first 24h, which remained elevated until day 5 (3.8±0.4 fold increase) (Figure 1A and Table S1). As for ferritin light chain, we found it to be regulated to a lesser extent by the infection. L-ferritin levels tended to decrease in infected BMM by 24h, and to be restored at day 3 of infection (Figure 1A).

We then tested if the increase in ferritin induced by infection leads to an increased diversion of iron into storage. BMM infected or not with *M. avium* were pulsed with ⁵⁵Fe ferric ammonium citrate and iron incorporation in ferritin was analysed in a native PAGE followed by exposure to autoradiography film. As observed in Figure 1B, infected BMM had a 40% increase of ferritin-bound- ⁵⁵Fe.

The expression of ferritins can be regulated post-transcriptionally by iron-regulatory proteins (IRPs). The binding of IRPs to the unique iron-responsive elements (IRE), which is present in the 5' untranslated region of both L- and H-ferritin mRNAs, blocks the mRNA translation [10]. Therefore, a decreased binding capacity of IRP could contribute to H-ferritin protein increase. Cytosols from control and infected BMM were tested for their IRP-IRE binding activity during the course of the infection. Results in Figure 1C show that the IRE-IRP regulatory system is kept at a low basal activity in both control and infected BMM over the 3 days post-infection. Moreover, inhibiting the cell transcription with actinomycin D blocked the

H-ferritin increase upon infection (Figure 1D), suggesting transcriptional, rather than post-transcriptional regulation by infection. To confirm this, we followed the expression of the genes coding for ferritin chains upon infection by qRT-PCR. *Fth1*, coding for H-ferritin, was found to be up-regulated by 5.2±1.9 fold in infected BMM at 24h (Figure 1E), correlating with the peak of protein expression (Figure 1A). The gene coding for L-ferritin (*Ftl1*), in accordance with the protein, was less regulated, with a reduction of 65% at day 2 of infection (Figure 1E).

2. Ferritin is regulated by infection independently of TNF-alpha or NO production

The infection of BMM with mycobacteria induces TNF-alpha production [17], a cytokine that (specifically) induces the expression of H-ferritin by an increase in *Fth1* transcription [18]. To investigate if the increase in H-ferritin observed during *M. avium* infection is a consequence of TNF-alpha production, we infected BMM genetically deficient in the production of TNF-alpha and followed the ferritin expression. As can be seen in Figure 2, the absence of TNF-alpha did not hamper the regulation of ferritin by infection, indicating that the increase in H-ferritin is not mediated by the production of this cytokine.

The infection of BMM with various mycobacteria also induces nitric oxide (NO) production [19]. Moreover, it has been recently reported that BMM exposed to exogenous NO can transcriptionally up-regulate ferritin expression [20]. To determine whether production of endogenous NO contributes to ferritin regulation during infection, we used BMM deficient in the NO synthase2 (NOS2). As shown in Figure 2, NOS2^{-/-} BMM exhibited similar ferritin protein profile changes to those of WT BMM post infection, demonstrating that NO is not involved in the regulation of ferritin by *M. avium*.

3. Ferritin regulation by *M. avium* infection is dependent on the activation of Toll-Like Receptor 2

Toll-Like Receptor (TLR)-2 is the main receptor for the recognition of several mycobacterial constituents [21]. To evaluate the role of TLR-2 in the regulation of ferritin by the mycobacteria, we used TLR-2^{-/-} macrophages and measured the cell ferritin content during the infection. Although TLR-2^{-/-} BMM increased H-ferritin in response to infection, this effect was markedly reduced in comparison with WT BMM. The absence of TLR-2 reduced by 62% the increase of H-ferritin after 24h of infection (Figure 2 and Figure 3A) and as much as 83% after 4 days (Figure 2). In accordance, TLR-2^{-/-} BMM did not increase *Fth1* mRNA expression upon infection (Figure 3B). Furthermore, L-ferritin was not regulated by the infection in TLR-2^{-/-} BMM (Figure 2). It could be argued that the engagement of TLR-2 might activate a pathway necessary, but not sufficient, to induce H-ferritin expression. To investigate if the activation of TLR-2 signalling is able, *per se*, to induce the increase in H-ferritin observed in *M. avium* infection, we used the synthetic ligand FSL-1 to specifically activate TLR-2 [22]. The stimulation of BMM with FSL-1 had a similar effect to the infection with *M. avium* (Figure 3C), strongly inducing H-ferritin, while slightly reducing L-ferritin content. These results show

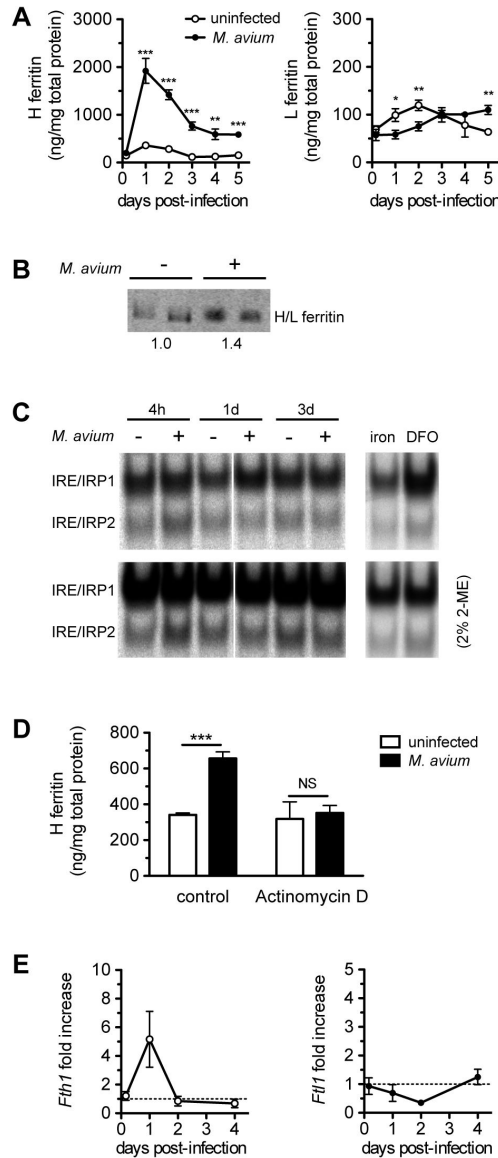


Figure 1. Effect of *Mycobacterium avium* infection on intramacrophagic ferritin. Bone marrow-derived macrophages were obtained from C57Bl/6 mice and infected with *M. avium*, as described in Material and Methods, or left uninfected. A - At different time points, macrophages were lysed and the amount of ferritin was quantified by ELISA. Data are presented as ng of ferritin per mg of total protein. The results are shown as average \pm SD from one experiment performed in triplicate out of four independent experiments. Superscripts indicate statistical significance between *M. avium*-infected and uninfected, within the correspondent time-point, as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. B - BMM uninfected or infected with *M. avium* for 24h were incubated for 6h with (^{55}Fe) ferric ammonium citrate. Total protein (18 μg) was loaded (in duplicates) in native PAGE and exposed to autoradiography to analyze protein-bound iron. A single band was detected corresponding to cytosolic H/L ferritin. The values indicate the average relative band intensity for each condition. C - BMM infected with *M. avium* for 4h, 1 and 3 days and respective uninfected controls were tested for IRP-IRE binding activity, by gel retardation assay. 2% of 2-mercaptoethanol (2-ME) fully activates IRP binding activity and shows equal loading. BMM treated with iron or deferoxamine (DFO) were tested in a separated gel to confirm the reliability of the assay. D - BMM were treated with the transcriptional inhibitor actinomycin D or with vehicle. After an 8h-infection with *M. avium*, the BMM were lysed and H- and L-ferritin were quantified by ELISA. Results show the average \pm SD from one experiment performed in triplicate out of three independent experiments. *** $p < 0.001$, NS not significant. E - At different time points, total RNA was collected from macrophages and the expression levels of ferritin genes was quantified by qRT-PCR, and normalized to *Hprt1*. Results are shown as fold increase in *M. avium*-infected macrophages in comparison with uninfected ones. Data are presented as average \pm SE from one experiment performed in triplicate from a total of two independent experiments.

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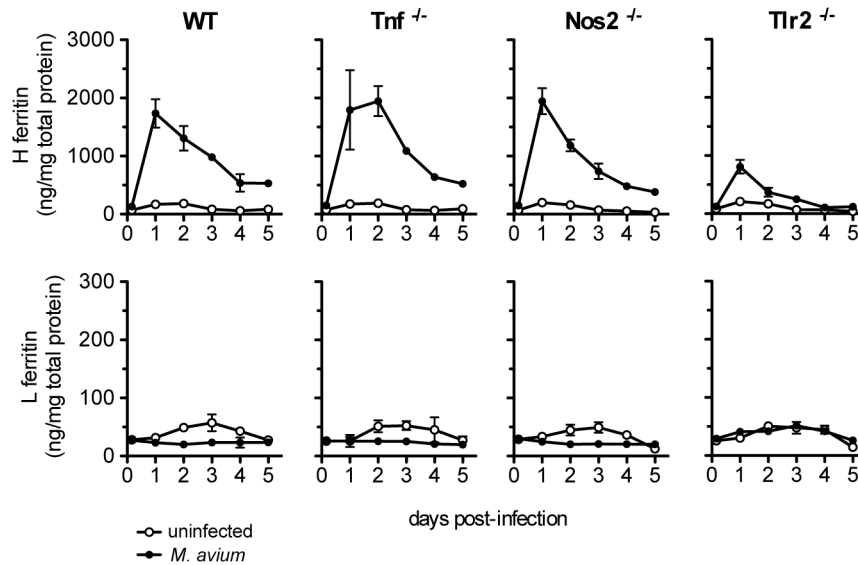


Figure 2. Effect of *M. avium* infection on ferritin content in the absence of TNF-alpha, iNOS and TLR-2. Bone marrow-derived macrophages were obtained from C57Bl/6 (WT), TLR-2^{-/-}, TNF-alpha^{-/-} and NOS2^{-/-} mice. BMM were infected and the ferritin content was quantified as described in Figure 1. The results are shown as average ± SD from one experiment performed in triplicate out of two independent experiments.

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that TLR-2 engagement is the main responsible for ferritin regulation during *M. avium* infection.

Discussion

Ferritin plays a major role in the control of iron distribution, and is regulated by iron, cytokines, hormones, growth factors and oxidants [9]. In the present work, we show that H-ferritin is induced in mouse bone marrow derived macrophages infected with *M. avium*. Previous studies had suggested that infection with intracellular pathogens can modulate ferritin expression. However, most studies were performed with immortal cell lines and the results were frequently in conflict with each other [23–27].

Our results, obtained with primary mouse macrophages, showed that the infection with *M. avium* increases the H-ferritin cell content, by acting at the transcriptional level. Furthermore, we showed that the infection did not lead to any observable change in IRE/IRP binding activity, likely because macrophages infected with *M. avium* produce lower flux of nitric oxide (a well-known activator of the IRE/IRP regulatory system) than macrophages infected with non pathogenic species [19]. Accordingly, elevated H-ferritin content was maintained in NOS2^{-/-} BMM infected with *M. avium* similarly to that of WT infected BMM. Our results further show that activation of TLR-2 increases H-ferritin through the activation of gene transcription, independently of NO. A recent report demonstrated that LPS (a TLR4 ligand) in combination with IFN-gamma strongly induced H-ferritin in IRP1^{-/-} BMM via endogenous NO production while H-ferritin content was maintained low in WT BMM [20]. Therefore, TLR-2 and TLR-4,

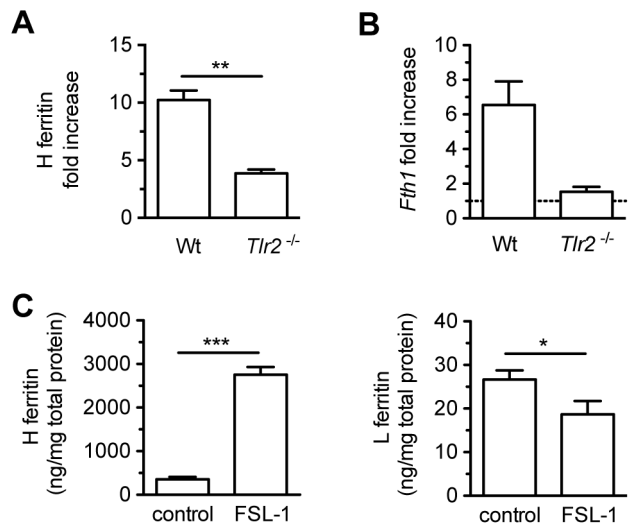


Figure 3. TLR-2 activation leads to increased expression of H-ferritin. A, B – BMM from C57Bl/6 (WT) and TLR-2^{-/-} mice were left uninfected or infected for 24h with *M. avium*. The H-ferritin fold increase in infected BMM in comparison with uninfected ones is shown at the protein level (A) and mRNA (B). C – BMM were treated with the TLR-2 agonist FSL-1 for 24h, and the levels of H- and L-ferritin was quantified by ELISA. Results show the average + SD from one experiment performed in triplicate out of three independent experiments. Statistical differences as described in Figure 1.

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which recognize different pathogen associated molecular patterns, are likely to have the same effect on ferritin, but adopting different pathways.

TLR-2 is the main receptor for the innate recognition of several mycobacterial constituents [21]. TLR-2 activation results in the killing of *M. tuberculosis* by NO-dependent mechanisms [28] and in bacteriostasis of *M. avium* [29] by mechanisms that remain elusive. Here, we found that TLR-2 engagement leads to H-ferritin increase, which may contribute to the anti-mycobacterial activity induced by TLR-2 engagement. Although we found that TLR-2 activation was sufficient for H-ferritin up-regulation, TLR-2^{-/-} BMM were still able to increase H-ferritin in response to *M. avium* infection, albeit to a much lesser extent. This suggests that other Pattern Recognition Receptors could have a minor role in the recognition of *M. avium* and consequent induction of H-ferritin.

M. avium infection had a much less pronounced effect on the levels of L-ferritin, overall resulting in the formation of ferritin proteins richer in H-chain. This may have important physiological consequences, since a higher H/L ratio has been shown to reduce the cell's labile iron pool [30]. Indeed, we found that *M. avium*-infected macrophages have a higher capacity for the incorporation of iron into cytosolic ferritin. Our results suggest, therefore, that H-ferritin up-regulation during infection may contribute to limit iron availability to mycobacteria, taking part of the macrophage nutritive mechanisms [31]. On the other hand, H-ferritin has also been implicated in protection against oxidative stress [32,33] and apoptosis [34]. We did not detect an increase in the levels of oxidative damage of macrophages upon infection with *M. avium* (Figure S1) and we can suggest that the observed increase in H-ferritin can contribute to the cytoprotection of the host cell. Further studies, including loss of function assays, are needed to obtain more definitive insights on the role of H-ferritin in the restriction of *M. avium* growth inside macrophages.

At a more systemic level, ferritin induction by microbial stimuli is likely to impair iron recycling by diverting it into storage, thus contributing to the development of the anemia associated with chronic disease (ACD) [35]. Several factors are involved in the development of ACD, including cytokines and hepcidin [36–40], but we have recently demonstrated that the anaemia observed during experimental mouse infection with *M. avium* is independent of hepcidin [41].

In summary, this work shows that the recognition of *M. avium* by macrophage TLR-2 leads to the increase of the expression of H-ferritin, through transcriptional activation, by mechanisms that although not completely elucidated, are independent of TNF-alpha and NO. TLR2 mediated-up regulation of H-Ft in

primary macrophages may be important in host protection against mycobacterial infections, by causing pathogen iron starvation and host cell protection and may also contribute to the development of ACD during mycobacterial infections. These findings can help better understand the tight regulation of host iron metabolism which occurs during the innate immune response to infections.

Supporting Information

Figure S1. Effect of *Mycobacterium avium* infection on the oxidation of peroxiredoxins (Prx). Bone marrow-derived macrophages were obtained from C57Bl/6 mice and infected with *M. avium*, as described in Material and Methods, or left uninfected. To evaluate oxidative damage, macrophages were lysed after 1 or 3 days and PrxSO₃, formed by the overoxidation of Prx, was detected by western blot. Uninfected macrophages exposed to 100 μM H₂O₂ for 20 min were used as a positive control. (DOCX)

Table S1. Effect of *Mycobacterium avium* infection on intramacrophagic ferritin. Bone marrow-derived macrophages were obtained from C57Bl/6 mice and infected with *M. avium* or left uninfected. At different time points macrophages were lysed and the amount of ferritin was quantified by ELISA. Day 0 refers to the time point immediately after infection. Data are presented as ng of ferritin per mg of total protein. The results are shown as average ± SD from one experiment performed in triplicate out of four independent experiments. (DOCX)

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Author Contributions

Conceived and designed the experiments: SSG CB MSG. Performed the experiments: SSG CB TS MSG. Analyzed the data: SSG CB TS PR RA MSG. Contributed reagents/materials/analysis tools: CB PS. Wrote the manuscript: SSG CB PR MSG.

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