THE IMPACT OF MACROPHAGE IRON EXPORT DURING *MYCOBACTERIUM TUBERCULOSIS* INFECTION

by

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(Under the Direction of FREDERICK D. QUINN)

ABSTRACT

Mycobacterium tuberculosis (Mtb) is still one of the major infectious diseases in the world with over 9 million cases and 1.5 million deaths in 2014. Iron is a crucial micronutrient for both mammals and bacteria, and upon infection, Mtb must fight the host for the same iron pool. Iron dysregulation in the host strongly associates with poor outcomes with several infectious diseases, including tuberculosis, AIDS and malaria, while inefficient iron scavenging by pathogens severely affects their virulence.

Hepcidin is the major regulator of iron serum levels in mammals, and promotes intracellular iron sequestration in hepatocytes and macrophages. In this dissertation, we first assessed the impact of Toll-Like Receptor (TLR) activation in iron homeostasis of human macrophages and elucidated the mechanisms of iron dysregulation in macrophages during infection. Here we show that TLR signaling induces hepcidin and downregulates ferroportin, promoting intracellular iron sequestration in human macrophages. Furthermore, here we reported that Hepc is highly expressed in human macrophages after TLR activation. This dissertation hypothesizes a novel mechanism by which Mtb circumvents the innate immune system, increasing intracellular iron bioavailability through induction of Hepc and downregulation of ferroportin expression in macrophages.

TB incidence has been declining worldwide, but is still a major public health concern in African and Asian countries. Furthermore, the increase in drug resistance cases calls for new therapeutic strategies to replace or complement currently available therapies. Recently, host-directed therapies showed promising results against Mtb, enhancing the effect of currently available anti-mycobacterial drugs, or directly decreasing bacterial replication. In this dissertation we show that IFNγ, which is associated with a protective immune response during tuberculosis infection, significantly inhibits pathogen-associated intracellular iron sequestration in macrophages and decreases iron availability to intracellular bacterial pathogens exposing iron dysregulation as an important factor during both innate and adaptive immunity against these pathogens. Thus, in the final chapters of this dissertation, I hypothesized that hepcidin inhibitors such as heparin or specific blocking antibodies significantly decrease intracellular bacterial replication during Mtb infection in human macrophages. Altogether, this dissertation uncovers macrophage iron export as an important host-directed therapeutic target during *M. tuberculosis* infection.

INDEX WORDS: Hepcidin, Ferroportin, Iron, Mycobacterium, Tuberculosis, TLRs, Heparin, IFNγ,

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DEDICATION

This work is dedicated to my nephews Pedro e Inês, for every birthday I missed over the past 6 years. I hope one day you read these words and understand the reason for my efforts.

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Reaching the final stages of this degree I fully recognize that a PhD is not fully the result of one's intellectual capacity or exceptional knowledge, but rather the outcome of extraordinary perseverance, stubbornness and resilience. Science, as any creative process of discovery, is ungrateful and frustrating. Many PhD students will agree that for every 10 experiments we perform only two or three give fruitful results, and without a supporting professional and personal environment no one would bear the failures and frustrations of a 6-year PhD. I'm no exception and I have to recognize that this degree is as much the product of my efforts as the support and help of many people around me. First of all, I want to acknowledge my mentors Dr. Fred Quinn and Dr. Pramod Giri. Thank you for your guidance and discussions, for showing me how to surpass my weaknesses and explore my strengths, and most of all for helping me grow as a scientist. Most sincerely, thank you very much "Bosses". I would also like to thank my committee members, Dr. Balaz Rada, Dr. Julie Moore and Dr. Roberto Docampo for your constructive criticism, for your ideas and suggestions that greatly improved this work.

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CHAPTER 1

INTRODUCTION AND SPECIFIC AIMS

Mycobacterium tuberculosis (Mtb) is still one of the major infectious diseases in the world with over 10 million cases and 2 million deaths in 2015. Iron is a crucial micronutrient for both mammals and bacteria, and upon infection, Mtb must compete with host for the same iron pool. Extensive literature has shown that Mtb mutants deficient in iron sequestration are severely attenuated, as well as on the host side, increased dietary iron or hemochromatosis have long been associated with a worse disease prognosis. Hepcidin (Hepc) is the major regulator of iron serum levels in mammals, and promotes intracellular iron sequestration in hepatocytes and macrophages. Furthermore, Hepc has been shown to be highly expressed during inflammation. It is then important to understand the inflammatory signals leading to increased Hepc expression in innate immune cells such as macrophages, along with the role of Hepc in the innate immune response against Mtb and other intracellular pathogens. Here we hypothesize a possible mechanism by which Mtb circumvents the innate immune system, increasing intracellular iron bioavailability through induction of Hepc expression in macrophages. With the intent to prove this, we will examine the following specific aims:

Aim 1: Determine the impact of different Pathogen Recognition Receptor's (PRR) signaling in Hepc expression and intracellular iron sequestration by human macrophages. TLR4 ligands (*e.g.* LPS) have previously been shown to induce Hepc. However, preliminary data shows that Pam3CSK4, a TLR2 synthetic ligand, does not induce Hepc expression in macrophages. We want to evaluate if Hepc induction is a general immune response to PRR's signaling or if other TLR ligands can have different effects on Hepc mRNA levels. The working hypothesis is that macrophages can distinguish intracellular from extracellular infection and recognize that intracellular iron sequestration is beneficial only in some specific inflammatory settings.

Aim 2: Comprehend the role of Hepc expression in macrophages during infection with intracellular pathogens. Hepc was first described as an antimicrobial peptide, but at concentrations far above those physiologically expected. It is not yet fully clear the exact role of hepcidin during infection, although recent reports seem to indicate a deleterious impact to the host during infection with intracellular pathogens. Preliminary data with BCG shows a clear induction of Hepc expression upon infection, leading to a decrease of its target ferroportin (FPN) and an increase in intracellular iron sequestration. The working hypothesis is that by inducing Hepc expression, Mtb can benefit from the resulting increased intracellular iron levels in the macrophage.

Aim 3: Evaluate the impact of Hepc chemical inhibitors in the course of infection by Mtb and other intracellular pathogens. As suggested in Aim 2, Mtb-mediated Hepc induction might lead to increased iron bioavailability in macrophages. Other labs have shown that capture monoclonal antibodies can reduce Hepc-mediated FPN degradation and prevent intracellular iron sequestration. Also, heparin has been shown to inhibit Hepc expression in hepatocytes, by blockage of BMP6 signaling. We hypothesize that inhibiting Mtb-mediated Hepc induction with heparin or blockage of Hepc function can prevent intracellular replication, and protect macrophages upon infection. We will also extend these conclusions to other intracellular pathogens such as *Listeria* sp. and *Salmonella* sp..

Mtb predominately infects lung macrophages, and it is well accepted within the field that an effective innate immune response might determine the course of infection[6]. In fact, upon infection most individuals are able to either clear or control bacterial replication with minimal damage to the host, although in approximately 10% of the cases the infected host fails to control pathogen growth and develops active tuberculosis (TB). Despite extensive research on the topic, the reasons behind Mtb activation are not yet fully known, but it is clear that both pathogen and host factors play a role in this process. Here we unveil a new factor that might contribute to increased Mtb susceptibility, and present a possible novel therapeutic approach targeting the host iron metabolism. Moreover, we expect that Hepc inhibitors or host iron modulators might be beneficial in a broader range of intracellular infectious agents and not only Mtb, as some preliminary data with *Listeria* and *Salmonella* seems to indicate.

CHAPTER 2

LITERATURE REVIEW

Tuberculosis epidemiology

Tuberculosis is one of the oldest diseases to afflict humankind, and might very well be the most lethal pathogen in our history. TB can be first found in recorded history as early as the 7th century BC by the name of consumption in the Middle East or *phthisis* in ancient Greece[1]. Hippocrates recognized TB as one of the major diseases in classical Greece both for its prevalence and mortality[2,3]. Recently, genetic studies have isolated *Mycobacterium tuberculosis* DNA from tissues of Egyptian mummies supporting the reports of skeletal tuberculosis in Egypt as early as 5000 BC[4].

Despite extensive efforts to eradicate Mtb through intensive screening and therapeutics programs, the World Health Organization (WHO[4–6]) reported over 9 million cases in 2014, with over 1 million fatalities, ranking as the leading cause of death in the world, side by side with HIV. Nevertheless, incidence has been slowly falling over the last 15 years at an average rate of 1.5%/year and prevalence is estimated to have fallen 42% from 1990 to 2015. Incidence is higher in Asia with 58% of total worldwide cases in 2014 where India, Indonesia and China account for 43% of the cases alone[4,5].

Besides Mtb infection alone, Mtb-HIV co-infection is another major public health concern. Out of the 9.6 million TB cases in 2014, more than 1 million were among HIV+ individuals from which about 35% resulted in death. Co-infection incidence rates are highest in the African region where over 30% of all TB cases are in HIV+ patients, and this region alone accounted for almost three quarters of worldwide HIV-TB co-infection cases[4,5].

Moreover, the cases of multidrug resistant TB (MDR-TB) and extensively drug resistant TB (XDR-TB) keep increasing due to lack of therapy compliance. In 2014, 3.3% of all new TB cases and 20% of previously treated cases were MDR-TB, accounting for a total of almost half million patients worldwide[4,5].

In addition to the high number of active TB cases, it is also important to mention the individuals with latent TB infection (LTBI) who are at risk of developing active infection. In the lack of better diagnostic tools, LTBI is identified by a positive immune response to Mtb antigens (PPD skin test or IGRA test) in the absence of TB disease clinical manifestation. A LTBI patient has an estimated 10% chance of developing active TB, but the risk increases in cases of HIV co-infection, direct first contact with active pulmonary TB disease or immunosuppressive treatment (α-TNF or transplant patients)[4,5].

In the United States TB incidence rates have been stably declining over the last decade to reach a national average of 3.0 cases/100 000 in 2013. This rate is still far from elimination which is set at < 0.1 cases/100 000[7]. Because of the great immigration rates in the USA, in 2013 approximately 13% of the total USA population was immigrant[8], and it is important to distinguish incidence in USA born and non-USA born citizens. In 2013 the national average among USA-born citizens was 1.2 cases/100 000, with the Southeast states and California showing the highest incidence rates. Among non-USA born citizens incidence rates are considerably higher reaching a national average of 15.4 cases/100

000[7]. In this case incidence rates are higher in the Northeast and Southwest states, which are also the states with higher immigration rates[8].

In summary, TB incidence has been declining worldwide and at particularly high rates in Europe and the United States. However, TB is still a major public health concern in African and Asian countries, and we are still far from achieving or even envisioning eradication. In today's globalized world where immigration rates to Europe and the U.S. are sky-high, MDR and XDR-TB is every country's problem and needs to be addressed globally. Better latent TB diagnoses are required to better estimate prevalence, and possibly identify susceptibility of reactivation, allowing health care practitioners to quickly start treatment and prevent TB spreading. In the same way, better treatment regiments can help decrease MDR and XDR-TB either by targeting new mechanisms and circumventing resistance or by shortening treatment length which would facilitate patient compliance.

Mtb clinical manifestations, pathology and treatment

Mtb is primarily a lung pathogen that persists in alveolar macrophages leading to extensive lung inflammation and pathology. TB symptoms are characterized by persistent cough that can last for several weeks, late day fevers (night sweet), constant fatigue, loss of appetite, and severe weight loss[9].

TB infection starts with inhalation of bacilli, transmitted by an active infected individual, and can progress in different stages depending on the host immune system (Fig 1). In primary TB infections, Mtb bacilli travel to the alveoli where they encounter alveolar macrophages and dendritic cells which actively phagocytize the bacteria and disseminate the infection to regional lymph nodes. This first stage can take 3 to 8 weeks and has no clear manifestation or transmission of disease. In 90% of primary infected individuals the host is capable of controlling and resolving the infection. In this case the bacilli are controlled by macrophages and dendritic cells in the lung, with a balanced pro and anti-inflammatory response. Bacterial replication is minimal and contained in small and invisible granulomatous structures until activation of adaptive immune cells. Clearance can take up to 3 years, but in some cases it never occurs and the pathogen goes into a life-lasting latent stage that can reactivate in case of immunosuppression[1].

In a second scenario that can last up to 3 months after primary infection, hematogenous dissemination of the bacteria leads to Mtb spread into the lower lobes of the lung and in some cases can cause systemic dissemination such as meningitis TB or military TB, which is in many cases fatal. This form of acute TB happens in 3% of infected individuals and is extremely hard to treat[1].

Mtb infection and spreading – molecular mechanisms of virulence

Mtb primarily infects alveolar macrophages and type II pneumocytes, so it’s natural that most of the molecular research until now has focused on virulence factors that impact invasion or replication in these cells. Traditionally, researchers focus in comparing Mtb with the attenuated BCG strain, in the hope that this would unveil the essential mechanism for bacterial pathogenesis[10]. Genetic analysis of Mtb lab strain H37Rv against attenuated *M. bovis* var BCG reveals 14 regions of differentiation (RD1-14), however most of these are already present in pathogenic *M. bovis* strain[11,12]. Out of these 14 regions, RD1, RD2 and RD14 are absent in *M. bovis* BCG and present in *M. bovis* and *M. tuberculosis* $H_{37}Rv$, indicating that these were lost over the attenuation process[10,13].

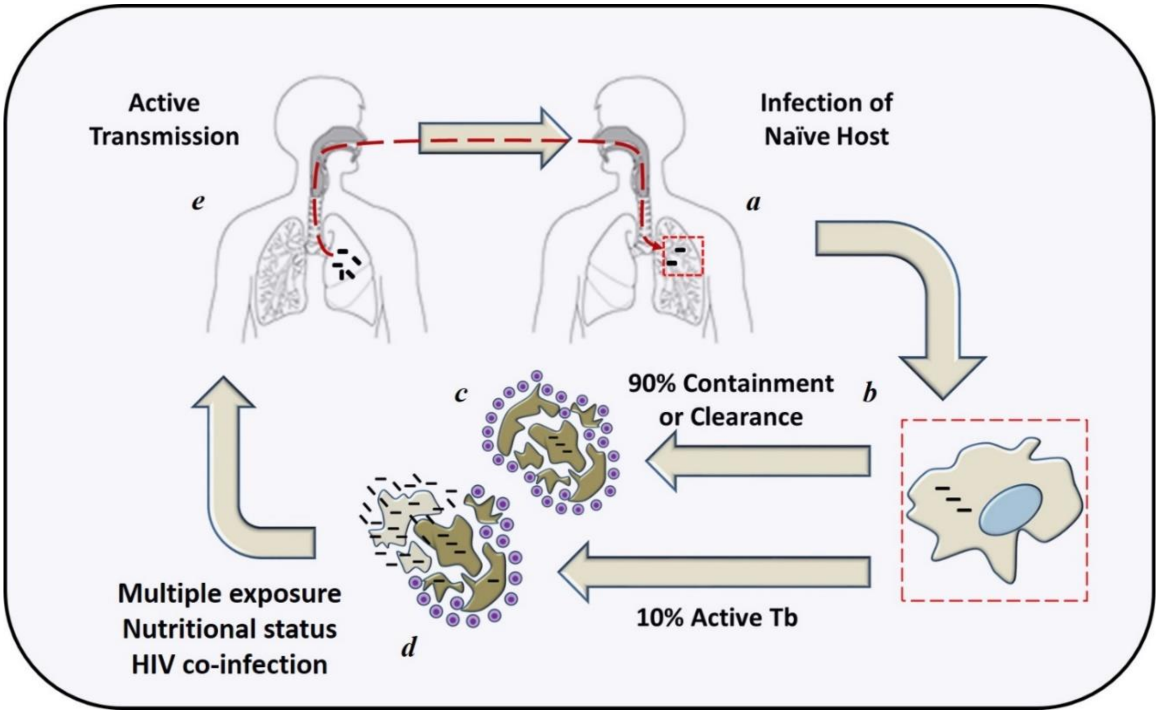

Fig 2.1: **Outcomes of Mtb infection.** Inhaled Mtb bacilli travel to the alveoli where they're phagocytized by alveolar macrophages (*a*). In 90% of the cases the host mounts an appropriate immune response controlling pathogen growth and replication with minimal pathology and tissue damage (*b*). In some cases, despite controlling bacterial replication, full clearance is not achieved and Mtb develops into latent stage inside small granulomas (*c*). When immunosuppressed, loss of granuloma integrity leads to Mtb reactivation and infection of the lower lobes (*d*). Uncontrolled bacterial replication augments lung pathology and initiates active aerosol transmission to the next host (*e*).

It is no surprise that efforts have been directed to proteins coded within these regions such as ESAT6/CFP10 (ESX-1) or PE/PPE (Proline-Glutamine/Proline-Proline-Glutamine) proteins. Since then multiple genes have been associated with Mtb virulence both *in vivo* and *in vitro*, which have been extensively reviewed in the literature. Just recently Forrellad *et al.* (2013)[14] published an exhaustive review on this subject.

Upon infection with a bacterial pathogen, resident macrophages engulf and phagocytize these pathogens. In an ideal immune response, phagosomes containing live bacteria will fuse with lysosomes from the Golgi apparatus, leading to an acidified environment, increased ROS and NOS species and high protease activity culminating in bacteria killing and clearance[15,16]. However, some pathogens, such as *Mycobacterium tuberculosis* or *Salmonella typhimurium*, are able to subsist and replicate inside macrophages by interfering with phagosome maturation and blocking the macrophage microbiocidal process[17].

Generally, pathogens resort to three different mechanisms to prevent phagosome killing: phagosome evasion, phagosome maturation arrest and oxidative and nitrosative stress neutralization.

Phagosome maturation arresting - Mtb has been shown to express several molecules capable of inhibiting or blocking phagosome maturation. Nucleoside diphosphate kinase (Ndk) is a 14 KDa Mtb-secreted protein, isolated from the culture media filtrate, with ATP and GTP binding activity. It has been shown to interact and inactivate Rab7 and Rab5 which are crucial for phagosome-lysosome fusion[18,19]. Finally, ΔNdk mutants show decreased intracellular replication in macrophages and increased phagolysosome co-localization[18].

Another Mtb protein that has been associated with phagosome-lysosome fusion inhibition is phosphotyrosine protein A (PtpA). This low molecular weight phosphatase can bind and block the host vacuolar H^+-ATPases and dephosphorylate a host vacuolar protein sorting protein (VPS33B) preventing phagosome acidification and phagosome maturation. Again ΔPtpA mutants show decreased replication in THP-1 macrophages, consistent with impaired phagolysosome evasion[20,21].

Finally, PE_PGRS30 has recently been added to Mtb's arsenal to prevent phagosome maturation. ΔPE_PGRS30 shows decreased virulence in mice, with decreased lung replication, inflammation and pathology, which is supported by increased lysosomal marker co-localization in macrophage models THP-1 and J774[22].

Phagosome evasion - For decades Mtb was believed to merely inhibit phagosome maturation growing and replicating inside this vesicular structure and never escaping to the cytoplasm[23–26]. However, recently Mtb has been associated with complete phagosome evasion through permeabilization of the phagosome membrane, just like *Shigella* or *Listeria*[27]. As described before, RD1 was one of the first virulence factors found in Mtb through comparison with attenuated *M. bovis* var BCG, although the mechanism was not understood. Recently ESAT6/CF10 proteins, secreted by ESX-1 T7SS, have been shown to have cell and membrane lysis properties, and are responsible for bacterial escape from the phagosome to the cytoplasm. However, this was observed only in dendritic cells and has not been reported in macrophages which are Mtb's preferential target[27].

Oxidative and nitrosative stress neutralization - Oxidative and nitrosative stress play a crucial role in bacterial clearance in macrophages. In the phagosome reactive oxygen species (ROS) and reactive nitrogen species (RNS) attack lipids, proteins and nucleic acids, culminating in bacterial death[17]. In order to survive and replicate in the phagosome, Mtb upregulates several antioxidant enzymes such as superoxide dismutase C (SOD C), catalase-peroxidase-peroxynitritase T (KatG) or thiol peroxidase (Tpx). SOD

C, responsible for detoxification of O^{2-} into molecular oxygen or hydrogen peroxide, is and outer-membrane lipoprotein upregulated by Mtb upon macrophage infection[17,28]. It is predicted that SOD C confers resistance to superoxide anions produced and pumped into the phagosome by the host macrophage, which is supported by *in vitro* attenuation of SOD C Mtb mutant in IFN-γ activated murine peritoneal macrophages but not on bone marrow derived MΦs (BMDMs) from respiratory burst deficient mice[29,30].

However, SOD C cannot fully protect Mtb from host oxidative stress[31] for which reason upon infection it also upregulates KatG, a catalase-peroxidase that degrades H_2O_2 and organic peroxides. ΔKatG mutant is severely attenuated in WT and iNOS KO mouse model but not in gp91 NADPH hindered mouse model[32], suggesting that KatG confers resistance to NAPDH-derived peroxides pumped into the phagosome. In addition, Mtb also expresses a TpX that reduces hydroperoxide and peroxynitrites both *in vitro* and *in vivo*[33–35]. Again ΔTpx mutants show decreased mortality and persistence in 57B/L mice when compared to WT strain, as well as decreased virulence in macrophages. However, the same phenotype is not observed in iNOS KO macrophages indicating that TpX is important in resistance against macrophage produced RNS[33].

Besides inducing antioxidant enzymes, Mtb’s cell wall also plays an important role in oxidative stress resistance. This hypothesis is backed up by mutagenesis of several genes putatively involved in cell wall synthesis which lead to increased susceptibility to ROS and NOS intermediates and decreased virulence in mouse models[36].

In sum, Mtb possesses many resources to counteract macrophage antimicrobial defenses and successfully inhibiting bacterial killing from early to late infection events. Here we have only focused on the best characterized virulence factors, although it’s important to notice that this is an area of intensive research, and new virulence factors keep being suggested and hypothesized. Recently much interest has been shown to how Mtb modulates macrophage phenotype (M1 *vs.* M2) and how it induces foamy cell/ gigantic cell formation[37,38].

Mtb and iron sequestration

Besides circumventing the host immune response and avoiding clearance, pathogens also struggle for essential nutrients inside the host. To replicate and grow inside the host Mtb must gain access to the macrophage's carbon, lipid and metals source. As so it is only natural that over the course of decades researchers have looked into genes and proteins involved in nutrient sequestration in hopes to create attenuated strains[39–44].

Iron is an essential cofactor required in the synthesis of the heme group of cytochromes involved in aerobic respiration, as well as hemeproteins required for amino acid and pyrimidine biogenesis and enzymes involved in the TCA cycle and DNA synthesis[45]. For such reason pathogens have evolved many mechanisms to recruit iron from the host iron pool, such as expression and secretion of siderophores. Siderophores are low molecular weight iron chelators, with higher iron affinity than the host's iron storage and transport proteins[46]. In Mtb the better characterized siderophores are mycobactin and carboxymycobactin, and the synthesis of these two lipophilic siderophores is dependent on proteins encoded by the *mbt* cluster. *ΔmbtB* mutants show defective growth and virulence in THP-1 macrophages when compared with WT stating the importance of iron recruitment in Mtb survival during infection[47]. Although, even these siderophores are able to sequester iron from the host, Mtb still needs to recover the iron bound to these high affinity chelators. *mbt-2* cluster genes encode IrtA and IrtB proteins, which are thought to assemble an ABC-iron transporter for the Fe^{3+}-siderophore complex[48–50]. Consistent with this hypothesis, *ΔIrtA/B* mutants also show reduced growth and replication in THP-1 macrophages, resembling *ΔmbtB* mutants. On the other hand, IdeR regulates iron uptake by downregulating *mbt* and *mbt-2* cluster genes to avoid iron toxicity[51].

Other proteins and mycobactin-independent mechanisms have been associated with iron sequestration[41,45,52], although definitive proof of how relevant these are in comparison to the mycobactin/carboxymycobactin mechanism is still missing.

Macrophage response to Mtb infection

As mentioned above Mtb is mostly a lung pathogen able to grow and replicate inside alveolar macrophages and dendritic cells. However, unlike other pathogens, Mtb does not seem to secrete any toxin and most of the disease’s pathology appears to be self-inflicted by the host immune system in an attempt to clear the bacteria. As so, it is believed that proper bacterial killing and clearance can only be achieved with a balanced pro- and anti-inflammatory response[53].

Macrophages (MΦ) are the primary target for Mtb replication, and over the course of decades, research focused on understanding how Mtb modulates macrophage antimicrobial activity[37]. MΦ are specialized innate phagocytic cells that respond quickly to pathogen or danger signals. These cells highly express PRRs like TLR, RLR or NLR that recognize a broad variety of Pathogen or Danger Associated Molecular Patterns.

Despite the tendency to group all macrophages by hematopoietic origin and physiological function, it is now clear that macrophages present an extraordinary plasticity to adapt to tissue environment and requirements [54,55]. In the lung resident alveolar macrophages (AMΦ) reside in the airspace juxtaposed to Type I and Type II pneumocytes, where they interact to maintain a balanced pro/anti-inflammatory environment that prevents infection without excessive pathology[56].

Regulatory signals

Because of the constant exposure to external antigens, the lung is, in steady-state, very anti-inflammatory just like other mucosa. AMΦs have low phagocytic activity (when compared with lung interstitial MΦ), secrete high levels of TGF-β and prostaglandins and have low CD86 surface expression and poor MHC-antigen presentation, which are all tolerogenic characteristics[57–60].

AMΦ express high surface levels of CD200R, which is an inhibitory receptor for AMΦ proliferation and activation. CD200L is expressed on the luminal side of Type II pneumocytes, and CD200R/L interaction might work as a detector of epithelial layer destruction and lung pathology for AMΦ[61–63]. When numbers of Type II pneumocytes decrease levels of CD200L also decrease and CD200R loses its inhibitory effect allowing AMΦ replication and activation[64–66].

AMΦ also express high surface levels of S1RPα which signals though SHP1 and leads to inhibition of phagocytosis, decreases TNF production and induces tolerance[67]. In the lung, S1RPα^{+} AMΦ recognize Surfactant Protein A and D (SP-A/D) secreted by alveolar epithelial cells to initiate signaling and promote an anti-inflammatory environment[68,69]. In addition, SPA and SPD also decrease complement activation and TLR signaling (see ahead for stimulatory signals).

Stimulatory signals

Toll-Like Receptors (TLR) are one of the major pathogen recognition receptors. Humans express up to 10 TLRs[70], but not all cells express all of these receptors[70,71] . Lung macrophages express high levels of TLR2, 4 and 9; however, levels of other TLRs increase upon ligand stimulation[72]. TLR1, 2, 4, 5 and 6 are expressed at the cell membrane surface and recognize bacterial surface or secreted ligands, while TLR3, 7, 8 and 9 are found in endosomes and recognize bacterial internal compounds such as DNA or RNA[73] (see Table 1 for localization, interaction and ligand specificity). Upon ligand binding TLRs signal through myeloid differentiation primary-response protein 88 (MyD88) adaptor molecule, except for TLR3 which interacts with TRIF, and lead to activation of NF-κB, induction of cytokine expression and upregulation of pro-inflammatory genes[73,74] (for detailed signaling description see Fig 2).

Until today TLR2 and TLR4 are the best characterized in Mtb infection. TLR2 can dimerize with TLR1 or TLR6 and recognizes lipoarabinomannan (Man-LAM)[75] and mycobacterial 19 KDa lipoprotein (lpqH), while TLR4 interacts with heat shock proteins 60/65 and atg38[72,74]. After TLR2 or TLR4 activation by Mtb agonists, signal starts with recruitment of adaptor molecules TIRAP and MyD88 and assembly of IRAK4/TRAF6 complex. TRAF6 can then activate TAK-1 which will phosphorylate NEMO and activate the NF-κB pathway. In parallel, TAK-1 also activates MAPK cascades, leading to phosphorylation and activation of JNK and p38 and finally activation of AP-1 transcription factor[72,73] (Fig 2). AP-1 and NF-κB induce expression of multiple inflammatory genes such as IL-6, TNF-α, IL-1, IL-12 and IL-18 (Fig 3).

The importance of TLR signaling in Mtb immune response has been shown in several different models, although it is still not definitive if it benefits pathogen or host. C57BL/6 MyD88 KO mice show a profound decrease in resistance to non-lethal Mtb infection, with increased mortality 5 weeks after infection and increased bacterial burden and lung pathology[76]. This lead to the hypothesis that TLR signaling is crucial for protection against Mtb, which is supported by other studies with TLR2 or TLR4 single KO[77]. Two independent groups showed that C57BL/6 TLR2 KO mice present increased susceptibility to Mtb in a high inoculum infection model, correlated to higher bacterial burden in the lungs[77,78]. However, these reports are somehow contradictory; Reiling *et al.* (2002)[77] hypothesize that TLR2 signaling is required to mount a proper Th1 inflammatory response, supported by the fact that TLR2-/- BMDM show decreased IL-12 and TNFα production *in vitro* in response to Mtb. On the other hand, Drennan *et al.* (2004)[78] associate TLR2-/- mice susceptibility to decreased macrophage RNI production and reduced microbiocidal activity which culminates in uncontrolled lymphocyte recruitment and activation and extensive lung inflammation and pathology.

TLR4 signaling is also implicated in Mtb immune response, but again its full extent and relevance is not yet clear. While some reports using C3H/HeJ mice with impaired TLR4 signaling, show no difference in resistance to non-lethal Mtb infection up to 30 weeks post infection[77,79] others point to a crucial role in TLR4 signaling to control chronic Mtb infection[80].

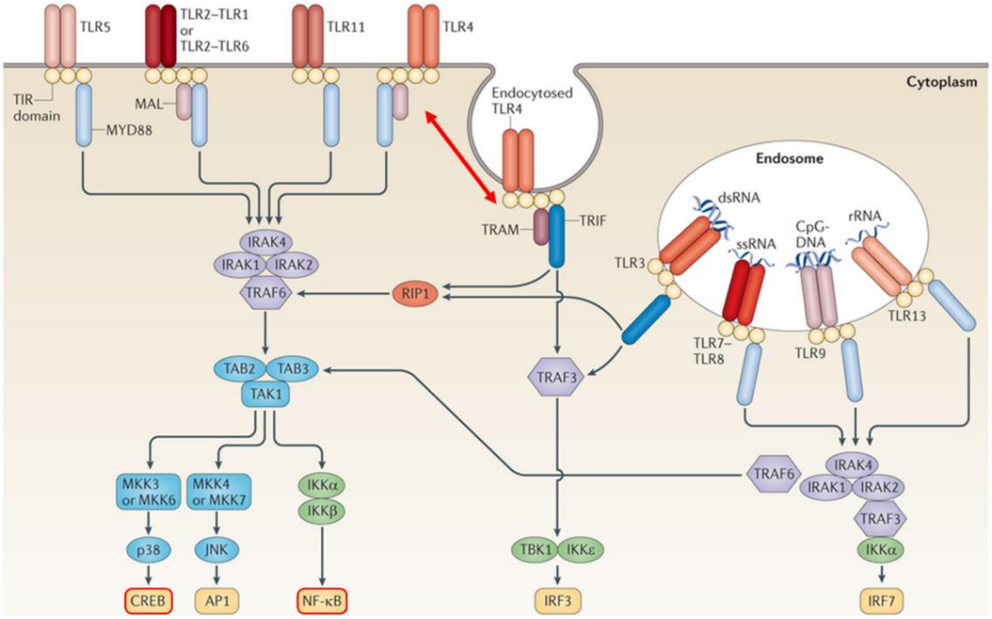

Fig 2.2: **Human TLR signaling.** TLR signaling is initiated by recognition of specific Pathogen Associated Molecular Patterns (PAMPs) (see Table 1). TLR5 and 2, which can dimerize with TLR1 and 6, signal at the cell membrane, and upon ligand binding, its Toll–IL-1-resistence (TIR) domains engage TIR domain-containing adaptor protein MyD88 and MYD88-adaptor-like protein (MAL). TLR7, 8 and 9 are expressed in the endosome and signal through an analogous MyD88 dependent mechanism. TLR4 can signal either at the membrane surface through a similar pathway, or in the endosome through TIR domain-containing adaptor protein inducing IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM) in a mechanism in all similar to TLR3. Engagement of adaptor molecules activates downstream signaling pathways centered in interactions between IL-1R-associated kinases (IRAKs) and the adaptor molecules TNF receptor-associated factors (TRAFs), activation of mitogen-activated protein kinases (MAPKs) JUN N-terminal kinase (JNK) and p38, and activation of transcription factors. NFκB and CREB (highlighted) are the transcription factors with several putative binding sites on Hepc’s promotor region. Adapted with permission from *Nature Reviews Immunology* (2013) **13,** 453–460[73]

Furthermore, other reports seem to indicate that TLR signaling plays no role at all in Mtb infection. B57BL/6 mice TLR2/4 double or TLR2/4/9 triple KO show no difference in survival to low dose Mtb infection and do not reproduce the observed phenotype of MyD88 KO mice[81,81].

In sum, all these reports seem to indicate a role of TLR2 and 4 signaling in Mtb infection, although its outcome might not be straightforward. TLR signaling might lead to a protective response in some specific settings and to disease progression in others, which leads to contradictory results in different infectious models. Furthermore, unreported conditions such as microbiome, diet and growing settings might have a great impact in disease progression and be responsible for the differences observed between research groups.

C-type lectins are calcium dependent glycoproteins with high glycan specificity and a crucial role in direct pathogen phagocytosis[82]. Although its expression is best characterized in dendritic cells, some of these C-type lectin receptors (CLR) are important in macrophage immunity and activation. The macrophage mannose receptor (MMR) is a monomeric transmembrane protein, with an extracellular domain containing eight carbohydrate-recognition binding sites important in Mtb uptake and phagocytosis[83]. MMR recognizes Mtb lipoarabinomannan (LAM)[84,85] and is predicted to signal through a putative cytoplasmic tyrosine domain, which phosphorylates and activates CDC42, RHOB, PAK or ROCK1, involved in actin reorganization, membrane invagination and phagosome formation[82,86,86]. Besides its role on phagocytosis MMR has not been associated with any other inflammatory signaling such as cytokine expression or phagolysosome fusion[82]. Direct phagocytosis is thought to be relevant only in early primary Mtb infection, when complement is absent of lung airspace, as so the full impact of MMR is still debatable.

Another CLR involved in Mtb recognition by MΦs is macrophage-inducible C-type lectin (Mincle). Mincle recognizes trehalose-6,6-dimycolate (TDM)[87], an abundant mycobacterial cell wall glycolipid, and recruits FcRy adaptor molecule to initiate signaling through phosphorylation and activation of Syk and CARD9 which will culminate in NF-κB activation and cytokine expression[88,89]. Despite being involved in Mtb recognition and pro-inflammatory cytokine secretion, there seem to exist contradictory reports about the importance of Mincle signaling in Mtb immune response. While *in vitro* studies show a clear role in anti-Mtb response[90–92], *in vivo* models show little to no impact in Mtb innate immunity and bacterial burden[93].

All in all, despite clear evidence showing the involvement of CLR in Mtb recognition, phagocytosis and innate immune response, it is still not clear what is the biological impact of this receptor in the outcome of TB disease. Further research exploring the mechanistic functions of CLR in Mtb infection as well as epidemiological data from individuals with altered CLR signaling might help shed light on the role of these receptors in the course of TB infection.

NOD-like receptors (NLR) are cytoplasmic PRRs with conserved nucleotide oligomerization domains (NOD/NACHT), a C-terminus leucine rich domain (LRR) and an N-terminus CARD or PYRIN effector domain[94]. NLRs signaling initiates inflammasome assembly required for IL-1β and IL-18 cleavage and maturation as well as regulation of cell death under infection[95]. Depending on the N-terminus domain, NLRs can be divided in 4 families: NLRA, NLRB (BIR domain), NLRC (CARD domain) and NLRP (Pyrin domain)[94].

The best characterized NLR, NOD2 - a member of NLRC family - is known to recognize cytoplasmic muramyl dipeptides from Mtb or other pathogens through the LRR domain[96]. This interaction leads to recruitment and activation of RIP2 kinase through the N-terminus CARD domain. RIP2 K63-ubiquitination recruits and activates TAK1-TAB2/3 which phosphorylates NEMO complex, culminating in K48-ubiquitination of IKB and release of NF-κB for nucleus translocation, culminating in a similar outcome to that of TLR stimulation[97].

Other NLRC molecules such as NLRC4 are activated by cytoplasmic flagellin which initiates polymerization of NOD/NACHT domains. NLRC4 activation can directly cleave and activate Caspase-1 which will end with cleavage of pro-IL-1β and pro-IL-18 into their mature forms. NLRP family members require the adaptor molecule ASC for full assembly and activation of the inflammasome leading to a similar outcome[97]. ASC dependent inflammasome also regulates cell death favoring pyroptosis and inhibiting apoptosis and autophagy[95,97,98].

During Mtb infection TLR and NOD2 signaling induce pro-IL-1β/IL-18 expression and NLRP3-asc inflammasome is responsible for cleavage of these cytokines and induction of pyroptosis[99]. If this leads to protection or efficient clearance is not yet fully known, although IL-1R KO mice are more susceptible to Mtb infection suggesting a possible protective role for NLR activation[100–102].

Rig-I-like Receptors (RLR) are cytoplasmic DNA/RNA sensing molecules involved in anti-viral response and Type I interferon upregulation[103,104]. Until now there isn’t much information on the role of RLR's during Mtb infection, although Manzanillo *et al.* (2012)[105] reported that Mtb activates some cytosolic DNA sensing molecules, perhaps RLRs, that signal through STING/TBK1/IRF3 pathway and induces Type I IFN expression. This was later shown to be dependent on C-GAS rather than RLRs, still, in this publication the possible role of RLR is not addressed at all[106].

To summarize, MΦs possess a broad spectrum of receptors that allow them to detect and respond accordingly to extracellular, phagosomal and cytosolic pathogens. Although, Mtb is able to signal and activate several of these PRR, leading macrophages to sometimes respond in an inappropriate way. Altogether, we may conclude from two decades of extensive research that PRR signaling is most definitely a complex pathway which outcome is not easy to predict. Some signals may be beneficial in early stages of infection, but deleterious at later stages; some might be protective alone but have no impact at all when combined; and some might even induce containment but prevent clearance. Further research is needed to fully understand this signaling network and help clarify how these molecules interact during Mtb infection.

Macrophage microbiocidal activity

MΦ effector functions have been recently reviewed by Weiss and Schaible (2015)[17]. Besides recognizing and phagocytizing pathogens, macrophages are responsible for killing and clearing invading organisms, and contribute to the recruitment and activation of the adaptive immune system.

Phagosome formation starts with FcR or CLR signaling which activates rac1, rac2 or Cdc42 GTPases and initiates actin polymerization[17]. After bacteria internalization, the phagosome goes through a series of fusion and fission events which correlate with its maturation stage. During its maturation process both phagosomal membrane and content are drastically remodeled; while membrane proteins change to promote interaction with early endosomes, late endosomes and lysosomes at different stages of maturation, the fusion with these vesicles promotes a gradual lumen acidification, highly oxidative and degradative[28].

The early phagosome is characterized by fusion with early endosomes in a Rab5A dependent process[29]. During this stage the phagosome is refractory to lysosome fusion, and the internal milieu is mildly acidic (6.1 to 6.5) which creates a poor environment for hydrolytic activity.

The small GTPase Rab5A is expressed both on early endosome and phagosome membranes, where it is activated by GAPVD1 (GTPase-activating protein and vPS9 domain-containing protein 1)[30]. GTP-bound Rab5A interacts and recruits SNARE proteins p150-hvPS34/EEA1 (early endosome antigen 1), which are responsible for early endosomal fusion[107,108]. Concomitantly three types of fission events can happen; a Rab11A/COPI dependent mechanism mediates protein recycling to the plasma membrane, while SNX1/2/4-VSP26A/29/35 promote cargo retrieval to the trans-Golgi network[28]. The third fission event is dependent on ESCORT and originates on membrane-associated cargo to intraluminal vesicles in a process in everything similar to that of multivesicular bodies. These vesicles transport ubiquitinated proteins marked for proteosomal degradation and determine the transition to the late phagosome stage in what some call of an intermediate stage[28].

Table 2.1: **Human TLR specificity and localization.** TLR signaling is initiated by recognition of specific Pathogen Associated Molecular Patterns (PAMPs). TLR1, 2, 4, 5, 6 and 9 recognize primarily bacterial specific ligands either at the cell surface or in the endosome. TLR3, 7, and 8 respond to viral PAMPs in the endosome, after phagocytosis. TLR 10 has only recently been discovered and it's not yet known how or where it signals. Despite the redundancy and cross-reactivity between different TLRs, specific synthetic or purified ligands are commercially available allowing the study of single TLR signaling.

TLR	PAMP	Ligand*		Localization
1/2	3-acylated bacterial lipoproteins	Pam3Csk4		Membrane
2	LAM Gram$^+$ bacterial cell wall	Pam2Csk4		Membrane
3	Viral dsRNA	Poly I:C		Endosome
4	Lipopolysaccharide Gram$^-$ bacterial cell wall	LPS (O111:B4)		Membrane Endosome
5	Flagellin	FLA-ST		Membrane
2/6	2-acylated bacterial lipoproteins	FSL-1		Membrane
7	ssRNA virus	R848	R837	Endosome
8	ssRNA virus		ssPolyU	Endosome
9	Bacterial methylated DNA	ODN2216		Endosome
10	??	??		??
Invivogen commercial specific ligands				

The late phagosome is characterized by a more acidic pH, ranging from 5.5 - 6, as a result of increased membrane expression of proton-pumping V-ATPases. This stage is also defined by high proteolytic activity and LAMP1 marker membrane expression as a result of fusion with Golgi vesicles or late endosome[109]. Another useful marker to distinguish early from late phagosomes is Rab5/7 expression. By a mechanism that is not yet fully understood, transition from early to late phagosomes is followed by decrease of Rab5 expression and increase of Rab7 on phagosomal membrane[110,111], although this switch is crucial for complete phagosomal maturation. Rab7 interacts with RILP and promotes the confluent movement of late phagosome and lysosome, which when in close proximity fuse through SNARE proteins VAMP7/8[111–113].

The phagolysosome is the mature stage of the phagosome and the ultimate microbiocidal organelle. This stage is characterized by high expression of V-ATPases which creates a highly acidic milieu (pH ≈ 4.5), increased proteolytic and hydrolytic activity, and extreme oxidative environment, which all contribute to pathogen killing and clearance[17]. Phagosome acidification impairs bacterial metabolism and increases proteolytic activity of host lipases, proteases, hydrolases, exo- and endopeptidases. In macrophages NOX2 NADPH oxidase releases O^{2-} to the phagosome lumen, where it can dismutate (through SOD) into H_2O_2, and then generate hydroxyl radicals, singlet oxygen, hypochlorous acid or chloroamines through myeloperoxidase activity[114]. Meanwhile in the cytoplasm increased expression of inducible nitric oxide synthase (NOS2) generates NO^- which can diffuse through the membrane to form nitrogen dioxide, peroxynitrite, dinitrogen trioxide, dinitrosyl iron complexes, nitrosothiols and nitroxyl[43,44]. These ROS and RNS along with host enzymes can directly target and damage the exposed bacterial cell wall proteins and lipids. Internal bacterial components become accessible after cathelicidins and β-defensins permeabilize and disrupt pathogen cell wall integrity[115].

Altogether these mechanisms lead to pathogen killing and the resulting degradation products will activate phagosome receptors such as TLR3, 7, 8 and 9, propagating the inflammatory response and further recruitment and activation of neighboring MΦs until complete pathogen clearance. Although, while this is the description of a perfect outcome where the host responds promptly and properly to pathogen invasion, some pathogens are able to interfere with these mechanisms and prevent clearance. In the case of Mtb, the virulent tools used to circumvent macrophage immunity have been previously described in the virulence mechanism section.

Host Iron metabolism

Iron is an essential element in all domains of life as an important cofactor for the synthesis and function of numerous proteins. In eukaryotic organisms iron is required for hemeprotein and iron-sulfur protein synthesis which play a role in multiple metabolic processes[116]. However, free iron is also extremely toxic due to generation of oxygen radicals by Fenton reaction, leading to lipid membrane, protein and nucleic acids damage[117]. In mammals, free iron levels are minimal; out of the 3-5 g of iron present in an adult human body more than 95% is associated with functional protein moieties and iron transport or storage proteins[118].

In normal homeostasis conditions, more than 60% of body iron is retained in heme moieties and hemoglobin in erythrocytes with a lifespan of approximately 120 days, translating in loss of 20 mg/day through erythroptosis[116]. Approximately 18 mg/day can be recycled during erythrophagocytosis of senescent erythrocytes by macrophages, but the remaining 10% must be recovered through dietary absorption, which must rapidly increase in case of hemorrhagic iron loss[118]. Dietary iron is absorbed by enterocytes in the duodenal mucosa through the DMT-1 metal symporter in the apical membrane, and exported to the blood stream by FPN expressed in the basolateral membrane[119]. In the blood stream, iron is transported by transferrin to hematopoietic and iron storage tissues where its uptake is mediated by transferrin receptor 1 (Trf1)[120]

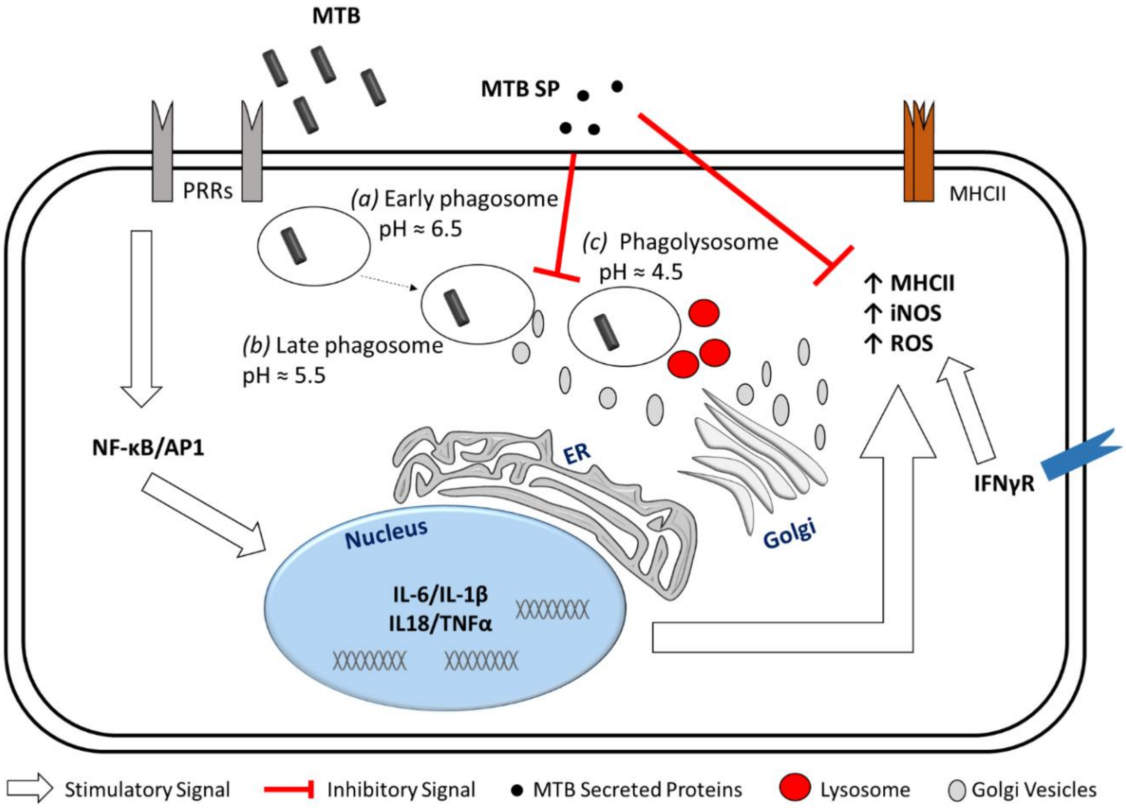

Fig 2.3: **Macrophage response to Mtb.** Upon infection, Mtb interacts with multiple PRRs expressed at the surface of AMΦs. Mtb interaction with MMR or FcR leads to bacterial phagocytosis and formation of early phagosome with mildly acidic milieu (pH ≈ 6.1 to 6.5) and poor hydrolytic activity (*a*). Fusion with Golgi vesicles increases expression of LAMP1 and V-ATPases, leading to vacuole acidification (pH ≈ 5.5 to 6) and increased hydrolytic activity of the late phagosome (*b*). In an ideal response, fusion with lysosomes will culminate in an extremely acidic environment (pH ≈ 5.5 to 6), highly oxidative and proteolytic (*c*). Stimulation of other

TLRs or IFNγ signaling can further increase microbiocidal activity through the increase of oxidative burst (iNOS and ROS). This signal also induces cytokine secretion and antigen presentation creating a strong pro-inflammatory environment. On the other hand, Mtb secreted proteins (LAM, ManLAM, PIM) can block phago-lysosome fusion, prevent vacuole acidification and decrease MHCII expression facilitating bacterial growth and intracellular replication.

In cases of systemic iron overload, iron levels are controlled by decreased dietary absorption, which is mediated by increased Hepc expression and consequent FPN downregulation[116,121]. As proposed by Ganz and Nemeth (2012)[122], increased iron levels induce Hepc expression and secretion in hepatocytes, which will then have both endocrine and autocrine/paracrine signaling. Endocrine signaling is characterized by binding of liver-produced Hepc to FPN expressed by macrophages and enterocytes, while autocrine/paracrine signaling is defined by the interaction of these two proteins in hepatocytes and Kupffer cells[116]. In both cases, Hepc promotes FPN internalization and degradation leading to decreased iron absorption and increased intracellular arrest in the liver and peripheral tissues[123].

Hepcidin induction mechanisms - Until today, the proposed model for iron mediated Hepc upregulation establishes BMP6 and SMAD signaling as the major effector molecules and Hemojuvelin (HJV), hemochromatosis protein (HFE) and transferrin receptors 1 and 2 (Tfr1 and 2) as sensors of increased extracellular iron levels[121]. In steady state, HFE interacts with high affinity with Tfr1, although saturated transferrin (holo-transferrin) competes with HFE for the same binding site. High holo-transferrin levels destabilize the HFE-Tfr1 complex and stabilize the formation of a similar complex with holo-transferrin-bound Tfr2[124]. This complex along with HJV, an iron-specific adaptor ligand of the BMP receptor, increases the sensitivity and potency of BMP6 signal[125,126]. It is not clear yet, but it's possible that intracellular iron sensors can regulate expression of BMP6 itself. On the other hand, in absence or low iron levels, Maraptase 2 (MT-2) is induced in hepatocytes leading to cleavage of HJV from the membrane surface, blocking the mechanism previously described[127].

Hepcidin inhibitors – Erythropoesis, hemorrhagic anemia and hypoxia are the major downregulator pathways of Hepc, consistent with the high iron demand of these processes. Although very little is known on the mediators and effectors of these pathways, hypoxia-inducible factors (HIFα) and BM-derived erythroid regulators are thought to be the major players in this mechanism[122].

Hepcidin in inflammation - Hepc was first described as an antimicrobial peptide isolated from blood and urine of chronically inflamed patients[128,129]. Despite its structural similarities with β-defensins, its microbiocidal properties were always mild and at concentrations far higher than those physiologically relevant[129]. After the discovery of Hepc's role in iron metabolism it was generally assumed that Hepc immune functions were mediated by the rapid decrease in iron availability to pathogens and responsible for the frequently observed anemia of infection[129,130]. This hypothesis is further supported by the fact that despite its protective role against extracellular pathogens, Hepc seems to promote replication of intracellular bacteria such as *Salmonella sp.*[131,132] or *Vibrio sp*.[133] The mechanisms leading to systemic Hepc induction during infection and inflammation are well described. IL-6 and other cytokine (IL-1β, TGF-β, IFN) signaling are crucial to endocrine expression by mouse hepatocytes, both in infection as well as sterile inflammation models[131]. However, induction of Hepc at the site of infection by resident macrophages and recruited leukocytes is not well understood.

Promotor bioinformatics analysis of the human Hepc gene (HAMP) reveals multiple putative regulatory transcription factors (TF), including hypoxia related factors (HIFα), BMP response elements (BMP-RE), and inflammation related elements (STAT3, NF-κB, IRF, Nrf2)[134]. Until now only BMP-RE2 and STAT3 have been validated experimentally and shown to bind and induce HAMP expression in hepatocytes both at a proximal (-200 bp) and distal (-2 Kbp) promotor regions[135]. TLR signaling has also been shown to induce HAMP expression in mouse macrophages, dendritic cells and neutrophils, yet it is not known if this is a direct result of TLR signaling or a feedback mechanism of increased cytokine secretion[136]. The existence of an NF-κB binding site would indicate that TLR signaling could directly regulate HAMP through activation of this TF. Nevertheless, despite the multiple studies connecting TLR signaling with Hepc induction, none has proven direct NF-κB binding to the promotor region of this gene[131,137,138]. Moreover, TLR mediated Hepc induction occurs at rather late timepoints supporting the hypothesis of a cytokine driven pathway[139].

Hepc and ferroportin in macrophage infection – Hepc induction during infection has been widely reported since the early 2000s, both *in vivo* mouse models and *in vitro* macrophage and hepatocyte models. Still, despite extensive research, it is not yet fully clear if Hepc induction leads to increased protection during infection, or is the result of pathogen driven immune modulation to facilitate replication[130,140]. Shortly after its discovery experimental data seemed to indicate that Hepc had protective functions during infection, both through direct microbiocidal activity as well as by decrease of iron availability to invading pathogens[129,141,142]. However, later studies looking at Hepc activity against intracellular pathogens point to the opposite conclusion[130]. Xu *et al.* (2010)[143] showed that FPN expression can inhibit HIV replication, and Hepc treatment could revert this effect both in macrophages and $CD4^+$ lymphocytes, but only when the cells were infected in the presence of iron. A similar effect was observed by two independent groups during *Salmonella* infection, where Hepc-mediated ferroportin degradation increased intracellular bacteria replication[144–146] . Besides these reports, other intracellular pathogens such as Mtb have been shown to greatly induce Hepc expression in macrophages and hepatocytes both *in vitro* and *in vivo* mouse models[139,147], although in this case it is not clear the positive or negative outcome of such response.

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CHAPTER 3

HOST-PATHOGEN INTERACTIONS AS NOVEL TARGETS FOR HOST-DIRECTED THERAPIES IN TUBERCULOSIS

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Abstract

Tuberculosis is one of the oldest diseases to afflict humankind, and *M. tuberculosis* (Mtb) might very well be the most lethal pathogen in our history. Over the past five thousand years that Mtb has infected our species, host and pathogen have evolved mechanisms and relationships that greatly influence the outcome of infection. Understanding this evolutionary race and how host-pathogen interactions impact bacterial clearance or host pathology leads the way to the rational development of new therapeutics that favor a host protective response. The fatiguing 6-month process of TB treatment, allied to the adverse side effects that can go from gastrointestinal disturbances to liver toxicity or peripheral neuropathy are major obstacles to patient compliance and therapy completion. The consequent increase in multidrug resistant TB (MDR-TB) and extensively drug resistant TB (XDR-TB) cases calls for novel therapeutic approaches, and host-directed therapies have recently showed promising results against Mtb, enhancing the effect of currently available anti-mycobacterial drugs or directly decreasing bacterial replication. Here we review the host-pathogen interactions during TB infection, how Mtb modulates and evades the host immune system and the currently available host-directed therapies that target each of these mechanisms. Rather than an exhaustive description of Mtb virulence factors, which falls outside the scope of this review, we will focus on the host-pathogen interactions that lead to increased bacterial growth or host immune evasion, and can be modulated by existing host-directed drugs. The host-directed therapies here reviewed may not be enough to contain and clear Mtb bacilli in an active TB patient, but might increase the effect of currently available anti-mycobacterial drugs, and give our immune system the little push it needs to efficiently contain latent TB infection.

Tuberculosis epidemiology

Tuberculosis is one of the oldest diseases to afflict humankind, and might very well be the most lethal pathogen in our history. Despite extensive efforts to eradicate Mtb through intensive screening and therapeutics programs, the World Health Organization (WHO) reported over 10 million cases in 2015, with almost 2 million fatalities, ranking as the leading cause of death in the world, surpassing HIV [1]. TB incidence has been slowly falling over the last 15 years at an average rate of 1.5%/year and prevalence is estimated to have fallen 42% from 1990 to 2015. Nonetheless, TB incidence remains high in Asia, India and Africa [2]. In addition to the high number of active TB cases, approximately one third of the world population is estimated to be latent infected (LTBI) and is at risk of developing active infection [3]. In the lack of better diagnostic tools, LTBI is identified by a positive immune response to Mtb antigens (PPD skin test or IGRA test) in the absence of TB disease clinical manifestation, and TBI patients have an estimated 10% chance of developing active TB. HIV co-infection, direct first contact with active pulmonary TB disease or immunosuppressive treatment (anti-TNF-α or transplant patients) significantly increases the risk of reactivation to 10% change every year [2]. Out of the 9.6 million TB cases in 2014, more than 1 million were among HIV+ individuals from which about 35% resulted in death, with higher incidence rates in the African region where over 30% of all TB cases are in HIV^+ patients [4].

Mtb is primarily a lung pathogen that persists in alveolar macrophages leading to extensive lung inflammation and pathology. TB symptoms are characterized by persistent cough that can last for several weeks, late day fevers (night sweat), constant fatigue, loss of appetite, and severe weight loss [1,5,6]. TB infection starts with inhalation of bacilli, transmitted by an actively infected individual, and can progress in different stages depending on the host immune system (Fig 1a,b). In primary TB infections, Mtb bacilli travel to the alveoli where they encounter alveolar macrophages and dendritic cells which actively phagocytize the bacteria and disseminate the infection to regional lymph nodes (Fig 1c). This first stage can take 3 to 8 weeks and has no clear manifestation or transmission of disease. In 90% of primary infected individuals the host is capable of controlling and resolving the infection. In this case the bacilli are controlled by macrophages and dendritic cells in the lung, with a balanced pro- and anti-inflammatory response (Fig 1d). Bacterial replication is minimal and contained in small and invisible granulomatous structures until activation of adaptive immune cells. Clearance can take up to 3 years, but in some cases it never occurs and the pathogen goes into a life-lasting latent stage that can reactivate in case of immunosuppression [7] (Fig 1e). In a second scenario that can last up to 3 months after primary infection, hematogenous dissemination of the bacteria leads to Mtb spread into the lower lobes of the lung and in some cases can cause systemic dissemination such as meningitis TB or miliary TB, which is in many cases fatal (Fig 1b). This form of acute TB happens in 3% of infected individuals and is extremely hard to treat [7].

Traditional research in Mtb virulence focused in the comparison of virulent strains with the attenuated BCG strain [8]. Genetic analysis of Mtb lab strain H37Rv against attenuated *M. bovis* var BCG revealed 14 regions of differentiation (RD1-14), out of which three (RD1, RD2 and RD14) are still present in *M. bovis* virulent strain that were lost over the attenuation process [9–12]. Within these regions, multiple genes have been associated with Mtb virulence both *in vivo* and *in vitro*, which have been extensively reviewed elsewhere

[12] and their exhaustive description falls outside the scope of this review. Rather, we will focus on the host-pathogen interactions that lead to increased bacterial growth and can be modulated by existing host-directed therapeutics.

TB treatment and drug resistance

TB treatment requires complex drug regimens for long periods of time leading to severe side effects. WHO guidelines recommend the treatment of newly diagnosed TB cases with a 6-month regimen of isoniazid, rifampicin, pyrazinamide, and ethambutol during the intensive phase (first 2 months) followed by isoniazid and rifampicin for continuation phase (next 4 months) [13]. In cases of previously treated TB cases with medium to low risk of multidrug-resistance the addition of streptomycin to the abovementioned drug regimen during the intensive phase is recommended, followed by a one-month regimen of isoniazid, rifampicin, pyrazinamide and ethambutol, and a 5-month regimen of isoniazid, rifampicin, and ethambutol [4,6].

The fatiguing process of TB treatment with daily dosages for the course of 6 months, allied to the adverse side effects that can go from gastrointestinal disturbances to liver toxicity or peripheral neuropathy are the major obstacles to patient compliance and therapy completion [2]. In 2013, only 86% of newly diagnosed TB cases were successfully completed and therapy completion rates haven’t improved since 2005. This lack of therapy compliance leads to an increase in multidrug resistant TB (MDR-TB) and extensively drug resistant TB (XDR-TB) cases. In 2014, 3.3% of all new TB cases and 20% of previously treated cases were MDR-TB, accounting for a total of almost half million patients worldwide [2]. This urges the development of new therapeutic strategies either through the development of new anti-mycobacteria drugs or host-directed therapies that promote a protective immune response [4]. Here we review the host-pathogen interactions during TB infection, how Mtb modulates and evades the host immune system and the currently available host-directed therapies that target each mechanism.

Mtb systemic dissemination

Mtb is primarily a lung pathogen, but can in rare cases cause systemic dissemination resulting in meningitis or miliary TB. Invasion and replication in lung epithelial cells (pneumocytes) is thought to be critical for Mtb systemic dissemination and unlike phagocytic cells, pneumocytes invasion is mediated by bacterial adhesins and bacterial mediated internalization [14–16]. Inhibiting invasion of Type II pneumocytes with heparin and heparan sulfate, or blocking HBHA function with neutralizing antibodies, efficiently prevents Mtb dissemination [17]. Furthermore, *M. bovis* BCG, the only currently available Mtb vaccine, efficiently prevents TB meningitis or systemic dissemination in children through unknown mechanisms.

Macrophage invasion during Mtb infection

Inhibiting pathogen invasion of the host cells is a frequent therapeutic approach for other respiratory pathogens. During Mtb infection this strategy is particularly difficult since the major cell target is also a crucial player for the host immune system. Alveolar macrophages actively phagocytize Mtb bacilli through multiple mechanisms, and the internalization pathway greatly influences the macrophage microbiocidal functions [18–20].

During early primary Mtb infection, direct Mtb phagocytosis is mostly mediated by C-type lectin receptors (CLRs) [21,22]. The macrophage mannose receptor (MMR) recognizes Mtb lipoarabinomannan (LAM) [23,24] and is predicted to signal through a putative cytoplasmic tyrosine domain, which phosphorylates and activates CDC42, RHOB, PAK or ROCK1, involved in actin reorganization, membrane invagination and phagosome formation [22,25–28]. Another CLR involved in Mtb recognition by macrophages is macrophage-inducible C-type lectin (Mincle) which recognizes trehalose-6,6-dimycolate (TDM), an abundant mycobacterial cell wall glycolipid [12,19,29]. In later stages of infection or in secondary infections, antibody and complement opsonized bacteria are phagocytized through Fc receptor (FcR) and complement receptor (CR3), signaling through a similar mechanism which promotes efficient bacterial killing and controls replication [20,22]. Despite the importance of these receptors in Mtb phagocytosis, the impact of each mechanism in the outcome of infections is not yet clear. Recently a tyrosine kinase inhibitor used in cancer therapy has been shown to modulate Mtb uptake and promote bacterial killing *in vitro* and *in vivo* [30,31]. Moreover, this drug was particularly effective in combination with anti-mycobacterial drugs, but the exact mechanism remains elusive. It is possible that a decrease in bacterial internalization in macrophages increases antibiotic access to Mtb, or that inhibition of one specific internalization pathway leads to an alternative uptake mechanism activator of microbiocidal macrophage functions. Currently, imatinib is the only tyrosine kinase inhibitor tested as a modulator of Mtb invasion, but other similar drugs presently in trials for cancer therapy [31] might have similar impacts or help clarify the exact mechanism behind bacterial control *in vivo*.

Granuloma formation and pathology

A hallmark of TB infection and pathology is the granuloma formation and dynamics. The granuloma is a compact organized immunological structure built of macrophages, monocytes, dendritic cells, neutrophils, epithelioid cells, foamy macrophages, and multi-nucleated giant cells, enclosed by T and B lymphocytes [32]. Disease progression results from complex remodeling of the granuloma structure with increased hypoxic necrotic centers rich in lipids and foamy macrophages that fail to control bacterial replication and lead to granuloma caseation [32]. The virulence factors leading to granuloma restructure and rupture are not yet well described, but ESX-1 secretion system, ESAT6 and TDM are known to play an important role in the initial steps of granuloma formation [12,33]. On the host side, TNF-α, IL-6 and complement (C5) are important for cellular recruitment and maintenance of granuloma structure. In the granuloma center, the predominant cell death pathway of infected macrophages is crucial for the outcome of infection. Predominant apoptosis controls bacterial replication by efferocytosis of infected macrophages [34]. In contrast, necrotic cell death results in bacterial leakage into the growth permissive extracellular environment and a characteristic cording phenotype that hamper phagocytosis by new macrophages [32,33,35].

Efficient bacterial control in the granuloma requires a balanced pro- and anti-inflammatory environment [36]. Anti-TNF-α therapy in autoimmune patients has been shown to increase risk of TB reactivation [37], however, excessive TNF-α leads to increased macrophage necrosis and results in granuloma caseation [34,38,39]. Central in the regulation of TNF-α expression during Mtb infection are pro-inflammatory eicosanoids such as leukotrienes and prostaglandins [40,41]. Excessive leukotrienes promote TNF-α and type I IFNs which result in increased necrosis cell death, granuloma caseation and cavity formation [41]. On the other hand, IL-1 signaling promotes apoptosis and induces prostaglandin expression which counter-regulates the function of type I IFN [40,41]. Non-steroid anti-inflammatory drugs, such as ibuprofen, induce expression of anti-inflammatory eicosanoids which significantly ameliorates pathology during TB infection *in vivo* with reduced bacterial load [42]. Similarly, leukotriene inhibitors such as zileuton used for asthma therapy, also reduce bacterial load in Mtb susceptible animal models [40,41].

Modulation of the host adaptive immune response

Despite the extensive research in tuberculosis it is not yet clear the ideal adaptive immune response leading to efficient control of bacterial replication and clearance with minimal tissue damage [19,43]. Mtb infects professional antigen-presenting cells with significant impacts in antigen presentation and activation the adaptive immune response. Mtb infected dendritic cells have decreased MHC surface expression and impaired antigen processing and presentation to CD_4^+ T cells [44,45]. Mtb delays priming of T helper cells, modulates cytokine secretion by macrophages to promote differentiation of Treg, and secretes decoy antigens that modulate the humoral response [46,47]. CD_4^+ T cell activation and differentiation into T_H1, with IL-2 and Il-12 and into T_H17 subsets, with IL-6, IL-1β and IL-23, is essential for Mtb containment [46]. Thus, the effector cytokines produced by these two T helper cell subsets have long been hypothesized as an effective immunomodulatory host-targeted therapy for tuberculosis. Despite the long recognized importance of IFNγ producing T_H1 CD_4^+ cells for an effective adaptive immune response

[48,49], direct IFNγ therapy produced controversial results in tuberculosis patients [50]. Initial studies with atypical tuberculosis patients, showed that IFNγ treatment in combination with standard anti-mycobacterial chemotherapy had no impact in sputum culture, but a pronounced effect in treatment completion rates and decreased lung lesion severity [51]. Although, a similar study with pulmonary tuberculosis patients produced no significant benefit in chest radiology results, despite the attenuation of general disease symptoms (fever) and increased rates of sputum smear conversion [50]. Furthermore, other direct cytokine therapies with IL-2 or IFNα also failed to produce conclusive beneficial results during tuberculosis infection [52] indicating that single direct cytokine therapy might not be the best host-directed approach for tuberculosis treatment. Recent studies highlighting the importance of multifunctional T_H1 cells capable of producing multiple cytokines (IL-2, TNF-α and IFNγ) might explain this discrepancy between the importance of some cytokines for an effective host immune response and the inefficacy of these same cytokines in clinical trials [53–55]. Another immunodulatory therapeutic approach focuses on Treg downregulation. Mtb infection promotes a tolerogenic immune response and the differentiation of Treg to facilitate bacterial replication [19]. GR1-specific antibodies and denileukin/diftitox efficiently deplete Treg proliferation and other myeloid-derived suppressor cells and significantly enhance anti-mycobacterial drugs effect [56,57]. This is a very active area of research particularly in anti-cancer therapy, but must be approached carefully because breaking host tolerance is frequently associated with severe autoimmune diseases.

Macrophage activation signaling

Innate immune cells like macrophages or dendritic cells recognize a myriad of pathogen or danger associated molecular patterns (PAMPS or DAMPS) [22]. Efficient microbiocidal functions in macrophages require activation of these stimulatory pathogen recognition receptors (PRR) such as Toll-like (TLR) or Nod-like (NLR) receptor [22]. Mtb evades and modulates PRR signaling to promote recruitment of permissive macrophages and manipulate the host adaptive immune response [18,19,58,59].

TLRs are abundantly expressed in human macrophages and crucial for early pathogen recognition during infection [60]. The relevance of TLR signaling for Mtb containment is still debatable [61–63], but it is widely recognized that virulent mycobacterial strains modulate and evade TLR signaling [18]. Non-pathogenic mycobacterial cell wall glycolipids (lipoarabinomannan) strongly activate TLR2 signaling inducing a strong pro-inflammatory response [64,65]. Contrastingly, similar molecules from pathogenic mycobacteria (mannose-capped lipoarabinomannan) do not activate TLR2 signaling or induce pro-inflammatory cytokines [18,24].

Mtb interferes with phagosome maturation, compromises phagosome membrane integrity [66,67] and some controversial reports show that completely escapes the phagosome and resides in the cytoplasm [68–70]. NLRs are crucial to recognize cytosolic PAMPS during bacterial infection and play and important role in induction of type I IFN and inflammasome activation [71]. NOD2 recognizes bacterial muramyl dipeptide fragments of the cell wall peptidoglycan in the cytosol to induce autophagy and pro-inflammatory cytokine production [72]. However, Mtb muramyl dipeptides are N-glycolyl modified and modulate NOD2 signaling to an alternative pathway leading to production of type I IFNs, which are not protective during Mtb infection [73]. Furthermore, type I IFNs antagonize IL-1β and IFNγ host-protective signaling [74].

The use of PRR ligand adjuvants is a particularly active area of research in vaccine development [75–77], but the use of specific TLR or NLR agonists might also be useful as a host-directed therapy. TLR2 activation with its specific ligand Pam2Cys rescues T_H1 cell exhaustion and significantly ameliorates diseases in chronically Mtb-infected mice [78]. Similarly, NOD2 and TLR4 activation significantly enhances the effect of standard anti-mycobacterial drugs isoniazid and rifampicin in Mtb-infected dendritic cells [72]. These studies, although preliminary, show the potential of direct PRR activation as an immunomodulatory host-directed therapy for tuberculosis. Nonetheless, such therapeutic approaches must proceed with care since dysregulated PRR signaling is frequently associated with loss of immune tolerance and development of auto-immune diseases.

Inhibition of macrophage microbiocidal functions

Alveolar macrophages are the preferential cell target for Mtb infection. In an ideal immune response, macrophages efficiently phagocytize and control bacterial replication. Phagosomes containing live bacteria fused with lysosomes from the Golgi apparatus, leading to an acidified environment, increased ROS and NOS species and high protease activity culminating in bacteria killing and clearance [21,64]. However, *Mycobacterium tuberculosis* can subsist and replicate inside macrophages by interfering with phagosome maturation and blocking the macrophage microbiocidal mechanisms [25]. Generally, Mtb resorts to three different mechanisms to prevent phagosome killing: phagosome maturation arrest, phagosome evasion, and oxidative and nitrosative stress neutralization.

Phagosome maturation arrest - Mtb expresses several molecules capable of inhibiting or blocking phagosome maturation and phago-lysosome fusion; *e.g.* nucleoside diphosphate kinase (Ndk), a 14 kDa Mtb-secreted protein and isolated from the culture media filtrate, interacts and inactivates Rab7 and Rab5 which are crucial for phagosome-lysosome fusion [12,20,79,80]. Similarly, phosphotyrosine protein A (PtpA), a low molecular weight phosphatase, can bind and block the host vacuolar H^+-ATPases and dephosphorylate a host vacuolar protein sorting protein, preventing phagosome acidification and phagosome maturation [81–83]. Aside from these, many other virulence factors have been associated with phagosome maturation or arrest and extensively reviewed elsewhere [12,20]. Until now, IFNγ activation and autophagy induction seem to be the most promising pathways to promote phagosome maturation and phagolysosome fusion [84–86]. *In vitro* macrophage activation with recombinant IFNγ upregulates FcR and CR3 surface expression [87–89], favoring phagocytosis of opsonized bacilli. As mentioned above, this phagocytic pathway promotes phagosome acidification and phagolysosome fusion. Vitamin D3 protective effect during tuberculosis has long been recognized through elusive mechanisms [90]. Now, we realize that VD3 induces cathelicidin expression in macrophages, an antimicrobial peptide important in phagosome maturation and phagolysosome fusion [91]. Likewise, Imatinib promotes phagosome maturation, lysosome fusion and induces autophagy [30] a naturally occurring cellular process for recycling and degradation of cytosolic content through vesicular engulfment and lysosome fusion [92]. During Mtb infection, live bacilli-containing phagosomes are redirected to the autophagy pathway reactivating lysosome fusion and bacterial killing [84]. Another possible target is the NAD^+-dependent histone deacetylase sirtuin 1 (SIRT-1), which was recently shown to be downregulated during

Mtb infection and important to control viral and bacterial replication [93]. Resveratrol is a phytoalexin present in grapes and berries, frequently commercialized as food supplement and a natural SIRT-1 activator. Resveratrol and a synthetic SIRT-1 activator induce phagolysosome fusion and autophagy, restricting Mtb growth *in vitro* and *in vivo.* Anti-mycobacterial drugs have been shown to promote autophagy and the development of autophagy inducers with minimal cell toxicity is a very active research area in host-directed therapies for viral and bacterial infections [84,94].

Phagosome evasion - For decades Mtb was believed to merely inhibit phagosome maturation, growing and replicating inside this vesicular structure and never escaping to the cytoplasm [23,46,95,96]. However, recently Mtb has been associated with complete phagosome evasion through permeabilization of the phagosome membrane just like *Shigella* or *Listeria* [68]. ESAT6/CF10 proteins, secreted by ESX-1 T7SS, have cell membrane lysis properties [97] and responsible for bacterial escape from the phagosome to the cytoplasm in dendritic cells [12,98,99]. Currently there are no prospective therapies to target cytosolic bacilli and prevent phagosome evasion, but modulation of the host ubiquitination machinery, inducing autophagy and activating cytosolic PRRs has been shown important for containment of other cytosolic pathogens [100,101].

Oxidative and nitrosative stress neutralization - Oxidative and nitrosative stress play a crucial role in bacterial clearance in macrophages. In macrophages, NOX2 NADPH oxidase releases O_2^- to the phagosome lumen, where it can dismutate (through SOD) into H_2O_2, and then generate hydroxyl radicals, singlet oxygen, hypochlorous acid or chloroamines through myeloperoxidase activity [102]. In the cytoplasm, increased expression of inducible nitric oxide synthase (NOS2 or iNOS) generates NO^- which can diffuse through the membrane to form nitrogen dioxide, peroxynitrite, dinitrogen trioxide, dinitrosyl ion complexes, nitrosothiols and nitroxyl [103,104] . In the phagosome reactive oxygen species (ROS) and reactive nitrogen species (RNS) attack lipids, proteins and nucleic acids, culminating in bacterial death [25]. In order to survive and replicate in the phagosome, Mtb upregulates several antioxidant enzymes such as superoxide dismutase C (SOD C), catalase-peroxidase-peroxynitritase T (KatG) or thiol peroxidase (Tpx). SOD C detoxifies of O_2^- into molecular oxygen or hydrogen peroxide [25,66], while the catalase-peroxidase KatG neutralizes the NAPDH-derived peroxides pumped into the phagosome and TpX grants resistance against macrophage produced RNS [105,105]. As mentioned before TNF-α has a controversial role during Mtb infection. *In vitro* studies with murine macrophages resembling early stages of infection, show that TNF-α-mediated iNOS and ROS induction significantly decreases Mtb growth [106]. Although, at later stages of infection, TNF-α induces necrosis of infected cells in the granuloma center leading to bacterial leakage and replication, making direct TNF-α cytokine therapy unsuitable for ROS and iNOS induction [34,38,40]. Thus, ROS and iNOS inducers with no impact in cell death are a promising host-directed therapeutic approach for TB. Metformin is an FDA approved anti-diabetes drug shown to induce mitochondrial ROS production in Mtb infected macrophages and decrease bacterial burden [107,108]. Furthermore metformin, has a positive anti-inflammatory impact decreasing Mtb-induced lung pathology [109], and positively regulates lipid metabolism (see below).

Modulation of lipid metabolism and macrophage phenotype

Mtb efficiently modulates macrophages glycolysis pathway, promotes ketogenesis and differentiation into permissive foamy cells [13]. Foamy cells are lipid droplet rich macrophages, characteristic of chronic inflammatory diseases and infections [110]. In macrophages, Mtb infection increases glycose uptake and redirects acetyl-CoA from the citric acid cycle to D-3-hydroxybutyrate synthesis, which signals through the anti-lipolytic G protein-coupled receptor GPR109A to induce lipid accumulation and lipid-body formation [13]. Furthermore, Mtb cell wall lipids such as oxygenated ketomycolic and hydroxyl-mycolic acid activate TLR2 and the scavenger receptor MARCO to induce cholesterol uptake, sequestration and lipid droplet accumulation [111,112], which can serve as carbon source for Mtb to persist in nutrient limiting conditions [32,110]. These findings uncovered the cellular similarities of Mtb infection with other host metabolic diseases such as type II diabetes or hyperlipidemia and open the way to the use of anti-diabetic drugs and statins as possible host-directed therapies during tuberculosis [35,40,109,113].

As mentioned above metformin decreases Mtb replication in human macrophages through increased ROS production and bacterial killing [108]. However, aside from its impact on macrophage oxidative state, metmorfin also reduces glycolysis efficiency, acetyl-CoA production and possibly ketogenesis in macrophages [114]. A parallel therapeutic approach focuses on hypercholesterolemia drugs such as statins that inhibit cholesterol synthesis and significantly decrease lipid accumulation [115]. Despite the initial promising results in animal models treated with statins and antimycobacterial drugs [116,117], a retrospective analysis with a national medical claim database failed to recognize any beneficial effect of this drug during tuberculosis infection [118]. More retrospective studies and controlled clinical trials should help clarify the relevance of lipid accumulation and foamy cell differentiation in tuberculosis, and understand if the currently available drugs for diabetes and hyperlipidemia can be effective host-directed therapeutics for tuberculosis infection.

Modulation of macrophage iron status

Iron is an essential element in all domains of life as an important cofactor for the synthesis and function of numerous proteins. Upon infection, Mtb must compete with the host for the same iron pool and mutant strains deficient in iron sequestration are severely attenuated *in vitro* and *in vivo* [119–121]. In contrast, increased dietary iron or hemochromatosis is strongly associated with a worse disease prognosis during tuberculosis infection [122]. Mtb promotes intracellular iron sequestration in macrophages through two TLR-dependent redundant mechanisms targeting the host iron regulatory proteins hepcidin and ferroportin (Abreu, unpublished data). Ferroportin is the only known iron exporter in mammals, highly expressed in macrophages, enterocytes and hepatocytes [123,124]. During iron overload or inflammation hepcidin secreted from macrophages and hepatocytes binds to ferroportin, leading to its internalization and degradation, and resulting in increased intracellular iron sequestration in macrophages, hepatocytes and enterocytes [125–127]. Mtb infection in human macrophages directly downregulates ferroportin expression through TLR2 activation, and TLR4-induced ER-stress leads to hepcidin secretion which further decreases surface ferroportin resulting in a significant increase in intracellular iron levels (Abreu, unpublished data). Iron chelation therapy is common strategy to avoid cardiac complications in hemochromatosis and thalassemia patients [128]. During Mtb infection in human macrophages, iron chelation with the FDA approved deferiprone or deferasirox significantly decreases intracellular bacterial replication (Abreu, unpublished data) [129,130]. *In vivo*, deferasirox intraperitoneal injection during intravenous *M. avium* infection significantly decreases bacterial burden in the spleen but not in the lung or liver [131]. In future studies, it would be interesting to see impact of iron chelation through different administration routs and during a more relevant aerosol Mtb infection model. Similarly, retrospective studies with hemochromatosis TB patients might unveil the interactions of iron chelation with standard anti-TB drugs regimen. Nonetheless, iron chelation therapy should be approached with care since it will exacerbate the anemia condition resultant of chronic inflammation. A therapeutic alternative to decrease iron availability to Mtb, and simultaneously decrease the associated anemic condition, is direct hepcidin inhibition [132–134]. Non-anticoagulant heparins significantly decrease hepcidin expression in hepatocytes [135–139], and heparin-mediated hepcidin inhibition decreases intracellular iron levels in human macrophages with pronounced effects in bacterial replication (Abreu, unpublished data). Furthermore, blocking hepcidin function with specific antibodies is currently being tested for treatment of anemia with promising results [140], and could be expanded as a host-directed therapy for TB. In fact, preliminary studies *in vitro* with human macrophages show that hepcidin blocking with a specific monoclonal antibody significantly decreases Mtb and other intracellular siderophilic bacteria replication (Abreu, unpublished data). Further *in vivo* studies will clarify the impact of hepcidin inhibition during Mtb infection, but the recent studies with other siderophilic bacteria strongly support the hepcidin-ferroportin axis as promising novel host-directed therapy for TB.

Concluding Remarks

TB incidence has been declining worldwide and at particularly high rates in Europe and the United States. However, TB is still a major public health concern in African and Asian countries, and we are still far from achieving or even envisioning eradication. In today's globalized world where immigration rates to Europe and the U.S. are sky-high, MDR and XDR-TB is every country's problem and needs to be addressed globally. Novel host-directed therapies can help decrease MDR and XDR-TB either by enhancing the effect of currently available anti-mycobacterial drugs, targeting new mechanisms and circumventing resistance or by shortening treatment length which would facilitate patient compliance. Over the past five thousand years that Mtb has infected humankind, host and pathogen have evolved mechanisms and relationships that greatly influence the outcome of infection. Understanding this evolutionary race and how host-pathogen interactions impact bacterial clearance or host pathology leads the way to the rational development of new therapeutics that favor a host protective response. The host immune response to Mtb is a complex network of pro- and anti-inflammatory signals, and it is now clear that targeting a single aspect of the immune response with increased pro-inflammatory signals is not sufficient to treat TB. Most of the promising host-directed therapies here presented target many host-pathogen interactions and in some cases seem to induce both pro- and anti-inflammatory responses. As example: heparin prevents Mtb invasion of pneumocytes and systemic dissemination, but also modulates macrophages intracellular iron levels, cytokine secretion and leukocyte recruitment ; Similarly metformin and vitamin D3 promote phagolysosome fusion and autophagy, while inducing anti-inflammatory cytokine secretion which prevents excessive lung pathology; and hepcidin inhibition decreases intracellular iron levels, but also decreases lipid body formation and modulates cytokine secretion in macrophages (Abreu, unpublished data). Altogether, these compounds counteract multiple virulence mechanisms used by Mtb to evade the host immune response and establish infection.

Host-directed therapies alone might never be enough to contain and clear Mtb bacilli in an active TB patient, but will certainly increase the effect of our currently available anti-mycobacterial drugs, and might give our immune system the little push it needs to efficiently contain latent TB infection.

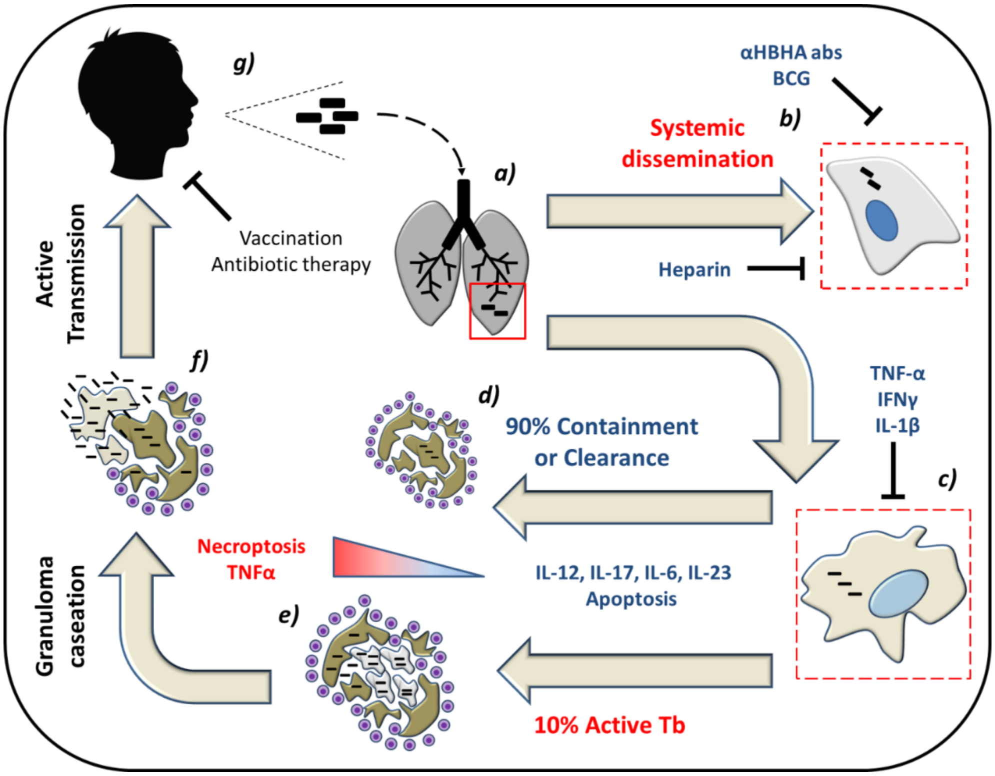

Fig 3.1: **Tuberculosis infection and transmission hallmarks.** Inhaled Mtb bacilli travel to the alveoli where they are phagocytized by alveolar macrophages (*a*). In rare cases, invasion of type II pneumocytes results in systemic dissemination and extrapulmonary TB, which can be prevented with *M. bovis BCG* vaccination or heparin treatment (*b*). In the lung, Mtb replicates in alveolar macrophages during early stages of infection (*c*) and in 90% of the cases the host mounts an appropriate immune response controlling pathogen growth and replication with minimal pathology and tissue damage resulting in bacterial clearance or a life-long latent stage with bacterial containment inside small granulomas (*d*). However, in 10% of the cases, an improper immune response or immunosuppression state results in loss of granuloma integrity, Mtb reactivation, dissemination and infection of the lower lobes (*e*). Uncontrolled bacterial replication and granuloma caseation (*f*) augments lung pathology and initiates active aerosol transmission to the next host (*g*).

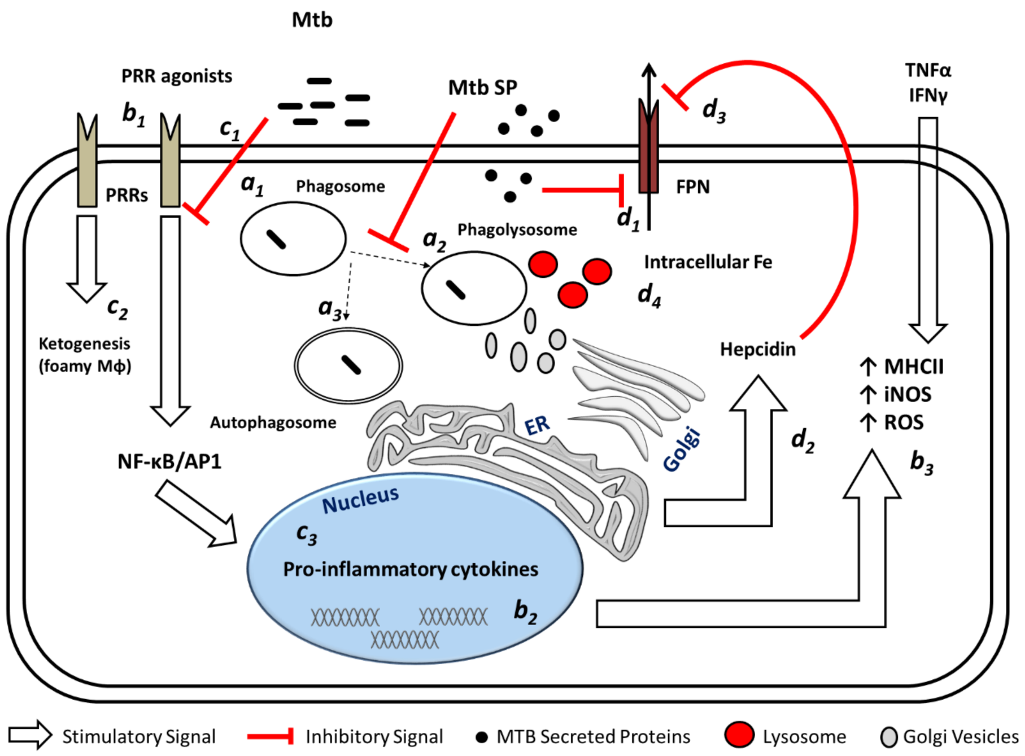

Fig 3.2: **Mtb modulation of macrophage immune functions.** Mtb is phagocytized by macrophages through different surface receptors (a_1) which greatly influence phagosome maturation and lysosome fusion (a_2). Mtb secreted proteins further inhibit phagosome fusion, but autophagy induction redirects immature phagosomes to the autophagosome (a_3) increasing bacterial killing. Macrophages detect pathogen invasion through activation of pathogen-recognition receptors (PRRs) (b_1) leading to expression of pro-inflammatory cytokines (b_2), increased reactive oxidizing species and activation of the adaptive immune system (b_3). However, Mtb cell-wall glycolipids modulate PRRs signaling (c_1), increase lipid accumulation, promote the differentiation in permissive foamy cells (c_2) and inhibit cytokine secretion (c_3). Mtb infection in macrophages directly decreases ferroportin transcriptional expression (d_1), and Mtb-induced ER-stress induces hepcidin expression and secretion (d_2). Secreted hepcidin binds to ferroportin leading to its internalization and degradation (d_3). Decreased surface levels of the iron exported ferroportin results in increased intracellular iron sequestration in macrophages (d_4) that can be redirected to the immature phagosome and used by Mtb for replication.

Table 3.1: Currently available host-directed therapies for tuberculosis

Compound	Host-pathogen interaction	Mechnism of action	Phase	Ref
M.bovis BCG **HBHA vaccines**	Systemic dissemination extrapulmunary infection	inhibits HBHA-mediated adherence and internalization of Type II pneumocytes	Aproved for human use Clinical optimization	Kohama et al . 2008, Pethe et al. 2001
Heparin	Systemic dissemination extrapulmunary infection	inhibits HBHA-mediated adherence and internalization of Type II pneumocytes	Aproved anticuagolant therapy	Menozzi et al. 1996,
	modulation of macrophage iron status	Inhibits Hepcidin exprecion and intracellular iron sequestration	Preclinical research and development	Abreu unpublished
Vitamin D3	macrophage anti-microbial functions	induces pahgolysosome fusion and autophagy in macrophages	Clinical optimization	Selvaraj et al. 2009
Metforin	macrophage anti-microbial functions	induces ROS and RNS prodution, reduces glycolisis and Mtb-induced foamy cell differentiation	Ready for clinical trials	Singhal et al. 2014, Viollet et al. 2012
Imatininb	modulates Mtb uptake	unknown	Preclinical research and development	Napier et al. 2011, Arora & Scholar 2005
	macrophage anti-microbial functions	induces pahgolysosome fusion and autophagy in macrophages		
PRR agonist	activation of adaptive immune system, macrophage anti-microbial functions, modulation of macrophage iron status	induces cytokine secretion, phagosome maturation, autophagy, ROS and RNS production	Clinical optimization	Chodisetti et al. 2014, Khan et al. 2016
Statins (rosuvastatins)	modulates macrophage lipid metabolism	inhibits chlesterol synthesis, lipid accumulation in macrophages and foamy cell differentiation	Ready for clinical trials	Turgeon et al. 2016, Parihar et al 2013, Skerry et al. 20114
Hepcidin blocking abs	modulates macrophage iron status	inhibits hepcidin-mediated ferroportin degradation and intracellular iron sequestration in macrophages	Preclinical research and development	Abreu unpublished
anti-TNFα	Decreases pathology and granuloma caseation	inhibitis necrosis of infected cells in the granuloma center	failed in trials	Wallis et al. 2015
Cytokine therapy (IFNγ, IL-17)	activates adaptive immune system	induces Th1 and Th17 adaptive immune response	Ready for clinical trials	Dawson et al. 2009, Johnson et al. 2003
NSAIDs (ibuprofen)	Decreases pathology and granuloma caseation	induces expression of of anti-inflammatory eicosanoids and apoptosis of infected cells in the granuloma center	Ready for clinical trials	Critchley et al. 2013
Zileuton (asthma drugs)	Decreases pathology and granuloma caseation	induces apoptosis of infected cells in granuloma center	Ready for clinical trials	Mayer-Barber et al. 2014, Wallis & Hafner 2015

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CHAPTER 4

ROLE OF THE HEPCIDIN-FERROPORTIN AXIS IN PATHOGEN MEDIATED INTRACELLULAR IRON SEQUESTRATION IN HUMAN MACROPHAGES[1].

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Key Points

- TLR signaling induces intracellular iron sequestration in macrophages through two redundant mechanisms
- TLR2 signaling down-regulates ferroportin transcriptional expression while TLR4 induces hepcidin secretion.

Abstract

Upon infection, pathogen and host compete for the same iron pool as this trace metal is a crucial micronutrient for all living cells. Iron dysregulation in the host strongly associates with poor outcomes with several infectious diseases, including tuberculosis, AIDS and malaria, while inefficient iron scavenging by pathogens severely affects their virulence. Hepcidin is the master regulator of iron homeostasis in vertebrates**,** responsible for diminishing iron export from macrophages during iron overload or infection. Hepcidin regulation in hepatocytes is well characterized and mostly dependent on IL-6 signaling during inflammation, although, in myeloid cells hepcidin induction and the mechanisms leading to intracellular iron regulation remain elusive. Here we show that activation of different TLRs by their respective ligands leads to increased iron sequestration in macrophages. By measuring the transcriptional levels of iron-related proteins e.g. hepcidin, ferroportin and ferritin, we observed that TLR signaling can induce intracellular iron sequestration in macrophages through two independent but redundant mechanisms. Interestingly, TLR2 ligands or infection with *L. monocytogenes* lead to direct ferroportin transcriptional down-regulation, while TLR4 ligands, such as LPS, induce hepcidin expression. Infection with *M. bovis* BCG promotes intracellular

iron sequestration through both hepcidin up-regulation and ferroportin down-regulation. This is the first study in which TLR1-9 mediated iron homeostasis in human macrophages was evaluated and the outcome of this study elucidates the mechanism of iron dysregulation in macrophages during infection.

Introduction

Iron is an essential trace element for the survival of virtually all organisms. This metal is required as a component of molecules sensing, transporting, and storing oxygen, as well as enzymes involved in oxidation and reduction of substrates during energy production, intermediate metabolism, and the generation of reactive oxygen or nitrogen species for host defense. During infections, prokaryotic, eukaryotic and viral pathogens use multiple complex mechanisms to acquire iron from their hosts, while hosts attempt to sequester it from pathogens, thereby starving them of iron and slowing their multiplication within the host[1,2]. Iron consequently represents a point of conflict between host and pathogen, and altered iron balance associates with poor outcomes in several infectious diseases, including tuberculosis[3], AIDS[4], and malaria[5].

Hepcidin, a 25-amino acid peptide hormone is the master regulator of iron homeostasis in vertebrates[6,6,7]**.** It is well known that diminished iron export from macrophages that recycle iron from senescent red cells rapidly induces hypoferremia due to the high iron demand of erythropoiesis. Excess levels of hepcidin have been recognized as the main cause of anemia of chronic disease[8,9,9,10]. Mechanistically, a decrease in iron efflux occurs when hepcidin binds with the iron exporter protein, ferroportin. Once bound, the complex is internalized and degraded culminating in decreased iron release from iron-exporting cells such as macrophages, hepatocytes, and duodenal enterocytes. Recently, Peyssonnaux, et al.[11] demonstrated endogenous expression of hepcidin by mouse myeloid cells, specifically macrophages, *in vitro* and *in vivo*. These myeloid cell types produced hepcidin in response to bacterial pathogens in a toll-like receptor (TLR) 4-dependent fashion. Conversely, bacterial stimulation of macrophages triggered a TLR4-dependent reduction in the iron exporter ferroportin[11].

Toll-like receptors are key sensors of the innate immune system[12]. TLRs recognize pathogen-associated molecular patterns (PAMPs) and control the hypoferremic host response. Lipopolysaccharide (LPS) is a cell wall component of Gram-negative bacteria recognized by TLR4. LPS injected into mice causes the release of pro-inflammatory cytokines and triggers a well-characterized acute phase response including induction of hepcidin by interleukin-6 (IL-6)[13–17]. Recently, it has been shown that the stimulation of the TLR2/6 pathway by Pam3CSK4 or FSL-1 synthetic triacylated lipopeptides triggers a profound decrease in ferroportin gene and protein expression in mouse bone marrow–derived macrophages independent of hepcidin[18]. Given the association of iron with infection outcomes, understanding how hepcidin itself is regulated during inflammation and infections is clearly important. However, most of the studies regarding the role of hepcidin and its regulation during infection or inflammation are conducted using hepatocytes. There is little information on how myeloid cells including macrophages regulate hepcidin expression and subsequently iron homeostasis when encountering pathogens or PAMPs.

In this study, we present a comprehensive evaluation of TLRs mediating iron homeostasis in THP-1 human monocytic cells. We determined that different TLRs regulate iron homeostasis in hepcidin dependent and independent manners. TLR2, TLR6 and TLR1/2 activation by Pam3CSK4, FSL-1 and Pam2CSK4, respectively, sequester iron by severely inhibiting the expression of ferroportin at the transcriptional level in human macrophages; interestingly, there was little change in hepcidin expression levels. Alternatively, TLR4, TLR7/8 and TLR9 restrict iron levels inside the macrophage by induction of hepcidin at the transcriptional and translational levels without altering the expression of ferroportin. TLR3 and TLR5 neither induce hepcidin nor reduce ferroportin expression in human macrophages. Collectively, this is the first study in which TLR1-9 mediated iron homeostasis in human macrophages was evaluated. The outcome of this study is useful towards an understanding of the mechanism of iron dysregulation during infection and with inflammatory diseases and disorders.

Material and Methods

Bacterial cultures

Mycobacterium bovis Bacillus Calmette-Guerin (BCG) Pasteur and *Mycobacterium tuberculosis* Erdman (Mtb) *(*generously provided by Dr Jeff. Cox (UC Berkley, CA USA) were cultured in 7H9 broth with 0.5% glycerol, 0.05% Tween 80 and 10% OADC at 37°C to OD of ≈ 0.8, aliquoted and stored at -80°C until used[19]. Samples were thawed at 37°C and plated on 7H10 agar for viable colony forming units (CFU/ml) enumeration. *Listeria monocytogenes* was obtained from ATCC (15313), cultured in BHI broth overnight at 37°C to OD of ≈ 0.6, transferred to BHI/20% glycerol, aliquoted and stored at -80°C until used[20]. Samples were thawed at 37°C and plated on BHI agar for CFU/ml estimation.

Cells and Cell culture

Human leukemia monocytic THP-1 cell line was obtained from ATCC (TIB-202) and maintained in RPMI with 2 mM L-glutamine and 10% FBS (C-RPMI) at 3-8 $X10^5$ cells/ml[21]. Myeloid differentiation factor 88 (MyD88)-deficient THP-1 cells (ΔMyD88) were obtained from Invivogen (San Diego, CA, USA) and cultured as the parental strain.

Human subjects and human peripheral blood mononuclear cells (PBMC) isolation

De-identified healthy human volunteers were recruited at the University of Georgia to donate venous blood. The studies were performed according to the guidelines of the World Medical Association's Declaration of Helsinki. Enrolled blood donors signed consent forms as described previously[22]. The human blood protocol (UGA# 2012-10769) and the associated consent form were reviewed and approved by the Institutional Review Board (IRB) of the University of Georgia. Coagulation was prevented with heparin and red blood cells were removed by Dextran sedimentation (GE Healthcare). The leukocyte-rich supernatant was washed two times in sterile PBS. PBMCs were isolated using a 5-step Percoll gradient centrifugation, collected from the 65% Percoll/PBS interphase and washed twice with PBS subsequently. Cell viability was >98% as assessed by Trypan Blue dye exclusion assay.

Chemical Reagents

Phorbol 12-myristate-13-acetate (PMA), synthetic diacylated (Pam2CSK4) and triacylated (Pam3CSK4) lipopeptides, polyinosinic-polycytidylic acid (poly I:C), *E. coli* O111:B4 ultrapure lipopolysaccharide (LPS), *Salmonella enterica* serovar Typhimurium flagellin (Fla-ST), synthetic lipoprotein FSL1, imidazoquinoline (R848) and Class A CpG oligonucleotide (ODN2216) were purchased from Invivogen (San Diego, CA, USA). Ammonium iron (III) citrate (FeAC) was obtained from ChemCruz (Santa Cruz Biotech, TX, USA).

Macrophage differentiation and Toll-Like Receptor (TLR) stimulation

For differentiation into a macrophage-like phenotype, parent and ΔMyD88 monocyte-like THP-1 cells were pelleted by centrifugation at 0.2Xg for 5 minutes, resuspended in C-RPMI with 50 nM PMA at $1X10^6$ cells/ml and seeded in 48-well tissue culture plates (Costar, Corning, NY, USA) for 24 hours at 37^{o}C and 5% CO_2. After differentiation, monolayers were washed twice with PBS to remove loosely adherent cells and rested overnight in C-RPMI 10%-FBS supplemented with 100 µM FeAC (unless otherwise stated) at 37^{o}C and 5% CO_2. After resting, macrophages were stimulated with different TLR ligands at concentrations described in Supplemental table 1.

For differentiation of primary human monocyte-derived macrophages (hMDM), we purified monocytes from hPBMC by plastic adherence and differentiated them into macrophages with M-CSF for 5 days as previously described[23,24].

Macrophage infection

Three hundred thousand monocyte-likeTHP-1 cells were differentiated as described above in 48-well plates and rested as described above in C-RPMI/FeAC overnight. The monolayers were then infected for two hours with BCG bacilli at a multiplicity of infection (MOI) of 10 (10 bacteria per host cell) and for one hour with *L. monocytogenes* bacilli at an MOI of five. The BCG-infected cells were incubated at 37°C for 72 hours and *L. monocytogenes*-infected cells for six hours in C-RPMI/FeAC with 50 μg/ml gentamicin. Fifty thousand human primary macrophages were infected as described above in 96 well plates with Mtb Erdman.

Isolation of mRNA and quantitative reverse transcription-PCR (qRT-PCR)

Total cellular RNA from 1X10^6 PMA-differentiated monocyte-like THP-1 cells was extracted with TRIzol (Invitrogen Thermo Fisher Scient., MA, USA) using the manufacturer's instructions and reverse transcribed into cDNA using the SuperscriptIII First strand cDNA synthesis kit (Invitrogen Thermo Fisher Scient., MA, USA) using poly dT_{20} primers. Quantitative PCR (qPCR) was performed using Bio-Rad IQ SYBR green supermix (Bio-Rad) in a iQ™5 Real-Time PCR Detection System. All values were normalized against GAPDH (ΔCT= CT [HAMP] - CT [GAPDH]). Fold change was calculated as $2^{-\Delta\Delta CT}$, where ΔΔCT= ΔCT (test sample) - ΔCT (control). The primer sequences for the genes examined were the following: human hepcidin (HAMP), forward, 5=-GGATGCCCATGTTCCAGAG-3=; reverse, 5=-AGCACATCCCACACTTTGAT-3=; human GAPDH, forward, 5=-GCCCTCAACGACCACTTTGT -3=; reverse, 5=-TGGTGGTCCAGGGGTCTTAC- 3=; human (ferroportin) FPN, forward, 5=-CACAACCGCCAGAGAGGATG-3=; reverse, 5=-ACCAGAAACACAGACACCGC-3=; Human ferritin (FTH), forward, 5=-AGAACTACCACCAGGACTCA-3=; reverse, 5=-TCATCGCGGTCAAAGTAGTAAG-3=; human IL-6, forward, 5=-CACAGACAGCCACTCACCTC-3=; reverse, 5=-AGCTCTGGCTTGTTCCTCAC-3=; human IL-8, forward, 5=-TCTGCAGCTCTGTGTGAAGGTG-3=; reverse, 5=-AATTTCTGTGTTGGCGCAGTG-3=.

Western blot

One million monocyte-like THP-1 cells were grown and differentiated in six-well plates, washed twice with ice-cold PBS and lysed with ice-cold immunoprecipitation (IP) lysis buffer for 30 minutes while placed on ice. Cell lysates were further disrupted manually by vigorous pipetting and vortexing. After centrifugation at 10,000Xg for 15 minutes at 4°C, supernatants were collected and stored at -20°C until analyzed.

Total protein concentration was determined by BCA protein quantification. Samples (20 µg) were mixed with Laemmli buffer (1x final concentration), and heated at 70°C for 10 minutes. Proteins were electrophoretically separated on a 15% sodium dodecyl sulfate (SDS)–polyacrylamide gel. Total protein was transferred to a PVDF membrane (Bio-Rad), which was then pre-incubated with blocking solution (5% nonfat dry milk in Tris-buffered saline containing 0.01% tween 20 [TBST], pH 7.4) for one hour, followed by overnight incubation with 1 µg of anti-ferritin1 rabbit monoclonal antibody (Cell signaling Tech, Danvers, MA, USA) and 1 µg anti-GAPDH rabbit monoclonal antibody (Cell signaling Tech, Danvers, MA, USA) at 4^{o}C. After primary incubation, the membrane was washed 3x with TBST and incubated for one hour with secondary anti-rabbit HRP conjugated antibody (Cell signaling Tech, Danvers, MA, USA).

All incubations and wash steps were performed at room temperature except otherwise stated. Cross-reactivity was visualized by using enhanced chemiluminescence (SuperSignalWestPico; Pierce) and quantified using QuantityOne application software (Bio-Rad, Hercules, CA).

Prussian blue staining

Four hundred thousand monocyte-like THP-1 cells were grown and differentiated in eight-well chamber microscopy slides as described above. After stimulation with TLR ligands, cells were fixed with 4% formaldehyde in PBS for 10 minutes at room temperature, washed with PBS and stained twice with a 1:1 solution of 4% hydrochloric acid and 4% potassium ferrocyanide for 25 minutes (Polysciences Prussian blue stain KIT). After washing with PBS, cells were counterstained with filtered 1% Nuclear Fast red solution for five to 10 minutes. After gentle washing with PBS and distilled water, slides were mounted and imaged with Axiovert 40CFL microscope and images were acquired with Axiocam MRC5 color camera 200X and 400X.

Immunofluorescence microscopy

Anti-ferroportin antibodies for surface and total ferroportin detection were kindly provided by Dr. Tara Arvedson (Amgen Inc., Thousand Oaks, CA, USA). Immunofluorescence staining was performed as previously described[25]. Briefly, 2 X 10^5 THP-1 cells were seeded and differentiated in eight- or 16-chamber microscopy slides and treated with TLR ligands as described above. For ferroportin staining, cells were incubated with 2 μg/ml mouse antibody diluted in C-RPMI. For detection, cells were incubated with 2 μg/ml goat anti-mouse labeled with alexa-fluor-488 (Invitrogen Thermo Fisher Scient., MA, USA). Incubation was performed at 4°C for two hours. Cells were gently washed 3X, fixed with 4% formaldehyde in PBS for 10 minutes, permeabilized with 0.1% Triton X-100 and counterstained with DAPI. Slides were imaged on a Zeiss Axiovert 200M microscope at 400X and 630X and images acquired with an Axiocam MRm grey scale camera.

Flow cytometry and intracellular staining

The mouse anti-human hepcidin antibody was kindly provided by Dr. Tara Arvedson (Amgen Inc., Thousand Oaks, CA, USA), and its specificity has been previously validated[26]. One million THP-1 monocytic cells were seeded and differentiated into 24-well plates, treated with TLR ligands as described above and with 1 µg/ml Golgi plug (BD Fisher) for another five hours to arrest Golgi transport. Cells were trypsinized, fixed, and permeabilized with Fix&Perm buffer (BD Fisher, Grand Island, NY, USA) according to manufacturer's instructions. Intracellular hepcidin was stained with 3 µg mouse anti-human hepcidin monoclonal primary antibody (mab2.7) for one hour, detected with goat anti-mouse alexa-fluor-488 (Invitrogen San Diego, CA, USA), and analyzed on a LSRII flow cytometer (BDbiosciences, San Jose, CA USA). All dilutions were performed in permeabilization wash buffer (BD Fisher, Grand Island, NY, USA). Data were analyzed with FlowJO FACS analysis software V7.6.5.

Intracellular labile iron pool quantification

The intracellular labile iron pool was measured using a calcein quenching assay as previously described[27] and adapted for flow cytometry analysis. Briefly, 1 X 10^3 THP-1 monocytic cells were seeded and differentiated in 48-well plates and treated with LPS or Pam3Csk4 up to 48 hours in iron-supplemented medium. At each time point cells were washed twice with warm PBS, stained with calcein-AM (Invitrogen Thermo Fisher Scient., MA, USA) for 15 minutes at room temperature, washed again with warm PBS, trypsinized, resuspended in FACS buffer and analyzed by flow cytometry before and after iron chelation with deferiprone (DFP). Quenched fluorescence was determined as percentage of Mean Fluorescence Intensity before iron chelation (xMFI) to 10 minutes after addition of DFP ($xMFI_{DFP}$) ($\frac{xMFI}{(xMFI_{DFP})} \times 100$). Cells grown in non-iron supplemented medium were used as negative controls.

Results

Iron increases LPS-mediated hepcidin induction

Hepcidin is regulated by two major pathways; iron metabolism and inflammation. To evaluate the interaction between these two pathways in human myeloid cells, specifically macrophages, THP-1 monocytic cells were differentiated into macrophage-like phenotype with PMA and stimulated with LPS for 24 hours in C-RPMI or in iron containing medium (DMEM-F12). Hepcidin mRNA levels demonstrated a 16-fold induction after LPS stimulation in C-RPMI, but interestingly this effect was further amplified up to 128-fold in DMEM-F12 when compared to the respective untreated control (Fig S1). To test if iron alone was responsible for this difference, we compared hepcidin basal mRNA levels in differentiated THP-1 cells in the presence or absence of iron supplementation, and observed that iron alone had no significant impact on hepcidin expression levels in THP-1 cells (Fig 1A). These results led to the conclusion that LPS mediated induction of hepcidin is more prominent in the presence of iron in cell culture medium, which more closely resembles the physiological conditions in mammalian serum. To confirm that these changes in mRNA levels translate into protein expression levels, we next assessed hepcidin concentrations in cells by intracellular staining. Our results indicated that LPS mediated hepcidin synthesis was more prominent in the presence of iron (Fig 1B).

It has been demonstrated that TLR2 recognizes lipoteichoic acid, peptidoglycan, and lipoproteins of Gram-positive bacteria[28]. In addition, TLR2 is well known for recognition of mycobacterial lipoproteins. To evaluate whether TLR2 activation induces hepcidin expression in myeloid cells similar to TLR4 activation by LPS, we stimulated macrophages with Pam3CSK4 (synthetic TLR2 ligand) in iron supplemented medium for 24 hours. Hepcidin mRNA levels showed that unlike LPS, Pam3CSK4 does not induce hepcidin expression in PMA-differentiated THP-1 monocytic cells (Fig 1C). Furthermore, intracellular hepcidin protein levels were similar between untreated and Pam3CSK4 treated cells (Fig 1D, E).

Activation of both TLR2 and TLR4 induces a signal through (MyD88), and TLR4 also can signal intracellularly through TRIF3[29]. Thus, we next assessed if LPS-mediated hepcidin induction is dependent on MyD88 mediated signaling. MyD88-deficient THP-1 cells were stimulated with LPS for 24 hours in iron supplemented medium. As shown in Fig 1E, MyD88-deficient macrophages demonstrated a significantly decreased hepcidin induction (two-fold compared to 64-fold in wild type macrophages) against the respective untreated control. It is interesting to note that MyD88-deficient THP-1 cells already showed decreased hepcidin basal levels and that after LPS stimulation demonstrated similar hepcidin mRNA levels as wild type untreated cells (Fig 1F).

TLR2 down-regulates ferroportin

Since hepcidin acts through ferroportin internalization and degradation, we next evaluated the impact of TLR2 and TLR4 signaling on ferroportin expression in macrophages. Our results showed that TLR4 ligand has no effect on ferroportin mRNA levels, while TLR2 activation by Pam3CSK4 significantly decreased ferroportin expression nearly three-fold (Fig 2A). This down-regulation corresponds to a significant decrease in surface ferroportin at protein levels similar to those resulting from LPS-mediated hepcidin expression (Fig 2B, C). Intracellular iron sequestration in macrophages is associated with decreased ferroportin levels in the cell membrane. To quantify the differences in total or surface ferroportin expression, differentiated THP-1 cells stimulated with Pam3CSK4 or LPS for 24 hours were surface stained and analyzed by flow cytometry. Consistent with our microscopic observations, regardless of its impact on hepcidin expression, TLR-2 activation significantly decreases surface ferroportin levels similarly to LPS treated cells (Fig 2D). Monocytic cell lines such as THP-1, may not always mimic the natural response of primary human macrophages. However, our data indicated with human primary monocyte derived macrophages (MDM) respond similarly to PMA-treated THP-1 macrophage-like cells in regard to TLR-4 mediated hepcidin induction (Fig S2A). Moreover, human MDM significantly induce hepcidin production in response to *M. tuberculosis* infection (Fig S2B). Future studies should expand the observations here reported with a human monocytic cell line to human primary myeloid cells and confirm the importance of hepcidin expression and intracellular iron sequestration in macrophages during infection with intracellular pathogens.

TLR2 signaling leads to increased intracellular iron sequestration

As discussed, hepcidin and ferroportin are key players in intracellular iron sequestration. Ferritin protein acts as a carrier for intracellular iron; thus, cytosolic ferritin is a good correlate of intracellular iron pool levels[30]. Because TLR2/6 directly down-regulate ferroportin, we hypothesized that addition of these ligands also would lead to intracellular iron sequestration and increased ferritin expression in macrophages. Differentiated THP-1 cells were stimulated with LPS or Pam3CSK4 for 24 hours and ferritin gene expression was assessed by qRT-PCR. Our data showed that activation of TLR2 and TLR4 significantly up-regulates (four to eight fold) ferritin expression compared to untreated controls (Fig 3A). Furthermore, ferritin mRNA levels also correlated with intracellular ferritin protein levels (Fig 3B, C).

To confirm that increased ferritin expression correlates with increased intracellular iron sequestration, Prussian blue staining was performed to assess total cellular iron content. Our results demonstrated that both TLR2 and TLR4 activation lead to intracellular iron sequestration (Fig 3D). Our results also confirm that TLR2 promotes intracellular iron sequestration by directly down-regulating ferroportin gene transcription, however, the molecular mechanism of TLR2 mediated ferroportin down regulation is unknown and needs to be investigated.

TLR2 inhibits ferroportin expression independent of hepcidin

To confirm that TLR2 activation mediates ferroportin down-regulation through a hepcidin-independent mechanism, the hepcidin gene (HAMP) was silenced using a hepcidin-specific lentiviral shRNA (shRNA-HAMP) (Fig 4A). Non-specific “Scramble” lentiviral shRNA (shRNA-SC) was used as a negative control. After achieving efficient lentiviral shRNA mediated gene silencing in THP-1 monocytic cell line, cells were treated with LPS and Pam3CSK4 for 24 hours and expression of ferroportin was evaluated by flow cytometry. Our results demonstrated that TLR2 activation equally down-regulates ferroportin expression in both control and hepcidin specific shRNA-silenced macrophages (Fig 4B). In contrast, TLR4 activation in hepcidin specific shRNA-silenced macrophages has no impact in surface ferroportin levels compared to untreated controls (Fig 4B). These results further indicate that TLR2 activation dysregulates iron homeostasis mainly by down-regulation of ferroportin gene expression in human macrophages.

TLRs signaling can induce hypoferremia through two independent pathways

To determine if the abovementioned iron sequestration mechanisms are restricted to only TLR2 and TLR4 activation by Pam3CSK4 and LPS, respectively, or if these can be expanded to the activation of other TLRs, PMA-differentiated THP-1 cells were stimulated with each individual TLR ligand for 24 hours and hepcidin, ferroportin, and ferritin mRNA assessed by qRT-PCR. According to the gene expression profile of hepcidin, ferroportin and ferritin, the TLRs could be grouped into hepcidin-inducers (TLR4, 7/8) and ferroportin downregulators (TLR1, 2, 6) (Fig 5A-F). Activation by TLR3, -5 and -9 does not induce hepcidin or downregulate ferroportin and has no impact on ferritin expression, thus were considered non-iron regulators (Fig S3). All TLR ligands except TLR3, -5 and -9 (poly I:C, Flagellin and ODN2216 respectively) induce ferritin expression up to three-fold. Furthermore, differentiated THP-1 moncytic cells treated with either TLR3 or TLR5 ligands show no increase in total intracellular iron levels or in the intracellular labile iron pool, as observed by Prussian blue staining and the Calcein quenching assay, respectively (data not shown). Nonetheless, despite having no impact alone, TLR3 activation can significantly downregulate ferroportin expression in combination with LPS, indicating that during infection even non-iron regulator TLRs (TLR3, -5 and -9) might play a role in intracellular iron sequestration (Fig S2).

Hepcidin expression in macrophages is independent of IL-6 signaling

Hepcidin expression during infection is mostly IL-6-dependent in hepatocytes[13,31]. Therefore, we speculate that after TLR stimulation, macrophages release pro-inflammatory cytokines such as IL-6 and IL-8 could be involved in TLRs activation mediated hepcidin expression. To explore this hypothesis, macrophages were treated with different TLRs for 24 hours as stated above, and gene transcription of IL-6 and IL-8 was evaluated by RT-PCR. Surprisingly, TLR1, 2, and 6, which do not induce hepcidin, showed 16 times more IL-6 expression then the untreated control, while TLR4 and TLR7/8 which do induce hepcidin, do not generate any changes in IL-6 expression levels (Fig 6C, D). In addition, IL-6 secretion in culture supernatants of macrophages treated with TLRs was consistent with IL-6 gene expression profile (Fig 6E-G). To further demonstrate that hepcidin induction is indeed IL-6 independent in macrophages, THP1 cells were stimulated with LPS and Pam3CSK4 in the presence of a IL-6 receptor-blocking antibody. As hypothesized, IL-6 blocking does not affect the hepcidin expression in response to LPS and Pam3CSK4 was unable to induce hepcidin expression in macrophages regardless of the presence of the IL-6 receptor blocking antibody (Fig 6H).

The cytokine IL-8 is frequently associated with early response to bacterial infection[32] and inflammatory hypoferremia[33], although a direct association between this cytokine and hepcidin has not been established. To determine if IL-8 is associated with TLR-mediated intracellular iron sequestration in macrophages, IL-8 mRNA levels were assessed by qRT-PCR in PMA-differentiated monocyte-like THP-1 cells stimulated with different TLR ligands. As expected, both LPS and Pam3CSK4 induced IL-8 to the same extent (approximately 7-fold induction) while Poly I:C, which has no impact on intracellular iron sequestration, promotes no changes in IL-8 mRNA levels compared to untreated controls (data not shown). These results demonstrate that iron sequestration in macrophages can be directly modulated by TLR signaling and the cytokines previously associated with hepcidin induction (IL-6 and -8) have a minimal impact.

Redundant role of hepcidin during intracellular bacterial infection mediated intracellular iron sequestration

To understand the biological relevance of the abovementioned mechanisms, the role of hepcidin in iron sequestaration during intracellular bacterial infection was determined. *M. bovis* BCG infection induced intracellular iron sequestration as measured indirectly by ferritin level (Fig 7C). BCG activates both TLR2 and TLR4 signaling[34], and likewise with pure ligands, BCG infection induces hepcidin expression and inhibits ferroportin expression to promote intracellular iron sequestration in macrophages (Fig 7A, B). However, infection with *L. monocytogenes* bacilli, a Gram-positive bacteria that proeminetly activate TLR2, does not induce hepcidin expression but downregulates ferroportin expression and subsequently promotes iron sequestration inside the macropahges (Fig 7E, F). Further, the role of MyD88 in hepcidin expression during BCG infection was assessed. As expected, when compared to the control parental strain, MyD88-deficient THP-1 cells have decreased of hepcidin expression 24 hours afterBCG infection (Fig 7D), confirming the role of TLR signaling in BCG-mediated hepcidin induction.

Discussion

In this study, we have shown that TLR activation with purified ligands leads to iron sequestration in human macrophages. Indeed, *M. bovis* BCG and *L. monocytogenes* PAMPs interact with TLRs and activate TLR-mediated signal transduction leading to increased iron sequestration in macrophages. The potential role of TLRs in regulating macrophage iron status, through ferroportin downregulation and hepcidin induction, two key regulators of iron homeostasis in humans, was explored.

This study presents for the first time a comprehensive evaluation of TLRs mediating iron homeostasis in differentiated THP-1 human monocytic cells. It has been shown that the macrophage iron modulatory response differs relative to the TLR stimulus provided. TLR 1, 2 and 6 activation by their respective synthetic ligands generates intracellular iron sequestration by directly downregulating iron transporter, ferroportin at the transcriptional level, while TLR4, and 7/8 activation increases hepcidin expression resulting in internalization and degradation of ferroportin in the proteasome[35]. Despite both mechanisms leading to increased intracellular iron levels and decreased extracellular iron availability, these constitute two independent yet redundant pathways activated during intracellular infection in macrophages.

Our results are in agreement with previously published study by Guida *et al.*[18] who demonstrated that TLR2/6 signaling induces hypoferrimia through a hepcidin-independent mechanism. In murine hepatocytes and macrophages, LPS treatment decreases intracellular iron sequestration both through hepcidin-dependent and -independent mechanisms[18,36], however, chronic TLR4 stimulation also prominently activates NRF2[37], which can counteract TLR4-supressed ferroportin expression[38].

Hepcidin controls the absorption of dietary iron as well as the distribution of iron between intracellular stores and extracellular fluids, including plasma[6]. During extracellular infections, increased hepcidin concentrations and associated hypoferremia are thought to be a host defense mechanism that decreases the amounts of iron available to extracellular microbial pathogens, thereby restricting their growth. While hepcidin-induced hypoferremia may protect against extracellular infections, hepcidin activity and associated shifts in iron compartmentalization may differentially affect pathogens that use alternative niches, such as intracellular *M. tuberculosis* and HIV. Kerkhoff and colleagues[32] conducted a cohort study and found that serum hepcidin concentrations increased with anemia severity and mycobacterial burden during HIV-*M. tuberculosis* co-infection. In this study, investigators generated multivariable Cox regression models, in which hepcidin was found to be an independent predictor of mortality in *M. tuberculosis*-positive, HIV-infected patients[39]. Therefore, one can speculate that hepcidin is intimately involved with the pathogenic processes occurring during HIV-associated tuberculosis, however the cellular mechanism of hepcidin expression in *M. tuberculosis*-HIV co-infection has not been fully investigated.

Expression of hepcidin by hepatocytes is stimulated by IL-6[13,14,31], and bone morphogenetic protein (BMP)[15,40] signaling pathways. Our data have shown that LPS-mediated hepcidin expression by human myeloid cells (macrophages) is not dependent upon IL-6 or BMP/SMAD signaling pathways. Indeed, it has been reported that murine macrophages did not increase hepcidin expression in response to BMP stimulation alone[41]. Our data are consistent with these earlier reports. Further investigation is needed to evaluate the cell regulatory pathways involved with hepcidin expression in human macrophages mediated by pathogens and PAMPs.

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Authorship

Contribution: R.A. designed research, performed research, analyzed data, and wrote the paper; F.Q provided guidance, wrote paper; P.K.G. designed research, analyzed data, provided guidance, wrote paper.

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Conflict-of-interest: The authors have no conflict of interest to declare.

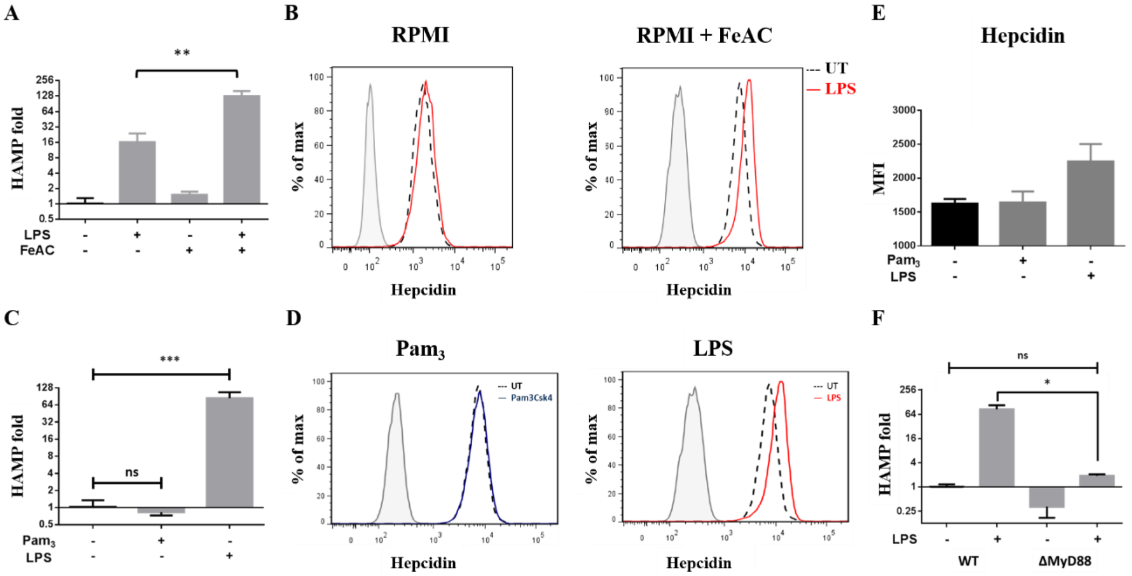

Fig 4.1: Iron increases TLR4-mediated hepcidin expression while TLR2 activation does not induce hepcidin expression. (A) THP-1 human monocytic cells were differentiated with 50 nM PMA and rested overnight in C-RPMI or C-RPMI supplemented with 100 µM ferric ammonium citrate (FeAC) and then stimulated for 24 hours with 500 ng/ml ultrapure lipopolysaccharide (LPS). (B) Differentiated THP-1 macrophages were treated as in A and stained for intracellular hepcidin using human anti-hepcidin antibody (mab2.7) and analyzed by flow cytometry. (C) Differentiated THP-1 macrophages were treated with TLR2 ligand (Pam3CSK4) or TLR4 ligand (ultrapure LPS) for 24 hours and hepcidin expression was quantified by qRT-PCR. (D and E) Macrophages treated as in C for 24 hours were stained for intracellular hepcidin using human anti-hepcidin antibody (mab2.7) and analyzed by flow cytometry. Hepcidin expression was quantified by mean fluorescence intensity (MFI). (E) Summary data from three independent experiments as represented in D. (F) Hepcidin expression in MyD88-deficient THP-1 (THP-1 ΔMyD88) macrophages treated with LPS as in A was measured by qRT-PCR. Hepcidin expression levels were analyzed by qRT-PCR and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as a control. **p<0.01, ***p<0.001. All data were from 3 independent experiments.

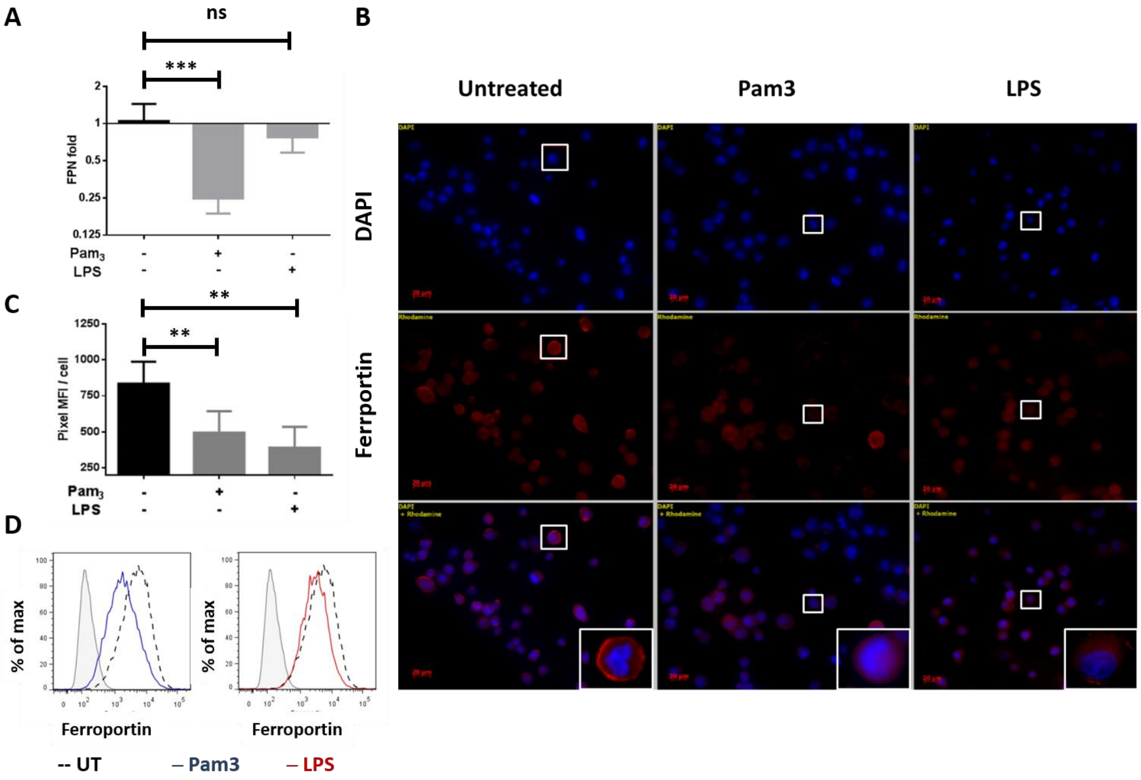

Fig 4.2: TLR 2 ligand inhibits ferroportin expression. (A) THP-1 macrophages were stimulated with TLR2 or TLR4 ligand and ferroportin expression was quantified by qRT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as a control (B) Surface ferroportin staining in THP-1 macrophages treated with TLR2 or TLR4 ligands was assessed by immunofluorescence microscopy. (C) Mean fluorescence intensity (MFI) was assessed from five microscopic fields in panel B. (D) Surface ferroportin expression in THP-1 macrophages treated with TLR2 or TLR4 ligands compared to untreated cells was assessed by flow cytometry. $^{**}p<0.01$, $^{***}p<0.001$. All data were from 3 independent experiments.

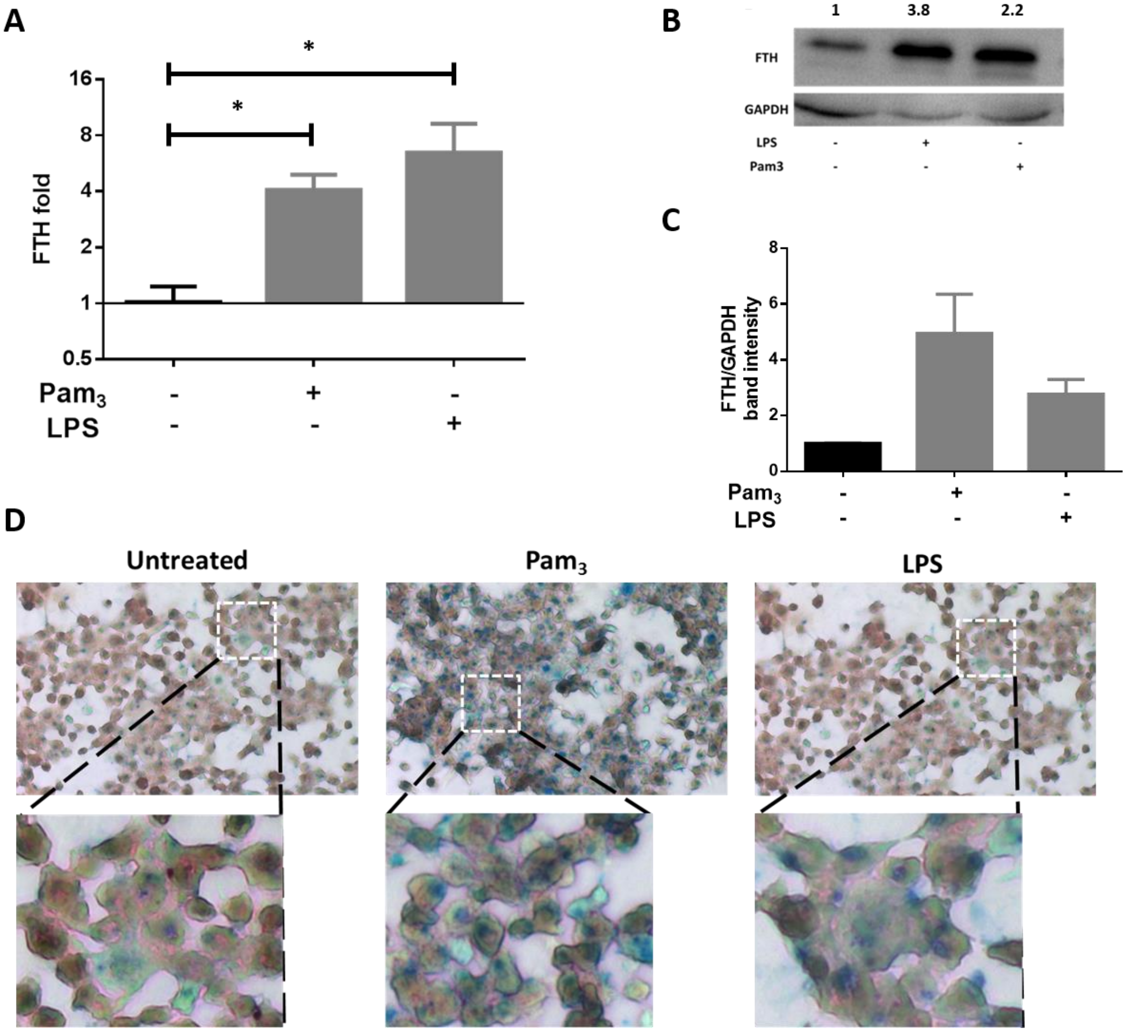

Fig 4.3: TLR2 induces intracellular iron sequestration though a hepcidin-independent mechanism. (A) Ferritin mRNA transcription levels from PMA differentiated THP-1 macrophages treated with TLR2 or TLR4 ligands for 24 hours quantified by qRT-PCR and normalized against untreated controls. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as a relative control. (B) THP-1 macrophages treated as in A and cell lysates were subjected to immunoblotting with the anti-ferritin (FTH) antibody. (C) Densitometry analysis from western blot in B. (D) Prussian blue staining of PMA differentiated THP-1 cells treated as described in A. Above panel shows low magnification field (10X) with high magnification (40X) on the lower side. *p<0.05. All data were from 3 independent experiments.

Fig4

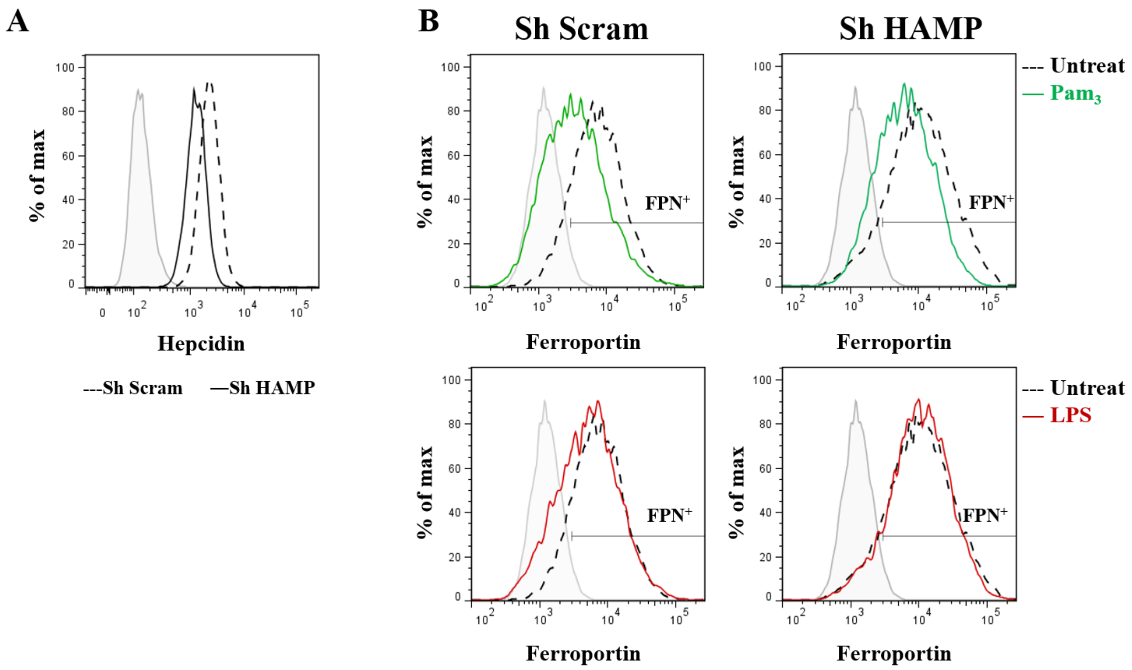

Fig 4.4: TLR2 inhibits ferroportin expression independent of hepcidin. Hepcidin gene silencing in THP-1 cells was achieved by lentiviral based shRNA transduction. Scramble short hairpin RNAs (Sh Scram) were used as a negative control. (A) Hepcidin expression in Sh Scram and hepcidin specific short hairpin RNAs (Sh HAMP) silenced THP-1 macrophages. (B) Surface ferroportin expression in Sh Scram and Sh HAMP THP-1 macrophages after Pam3CSK4 or LPS treatment for 24 hours. All data were from 3 independent experiments.

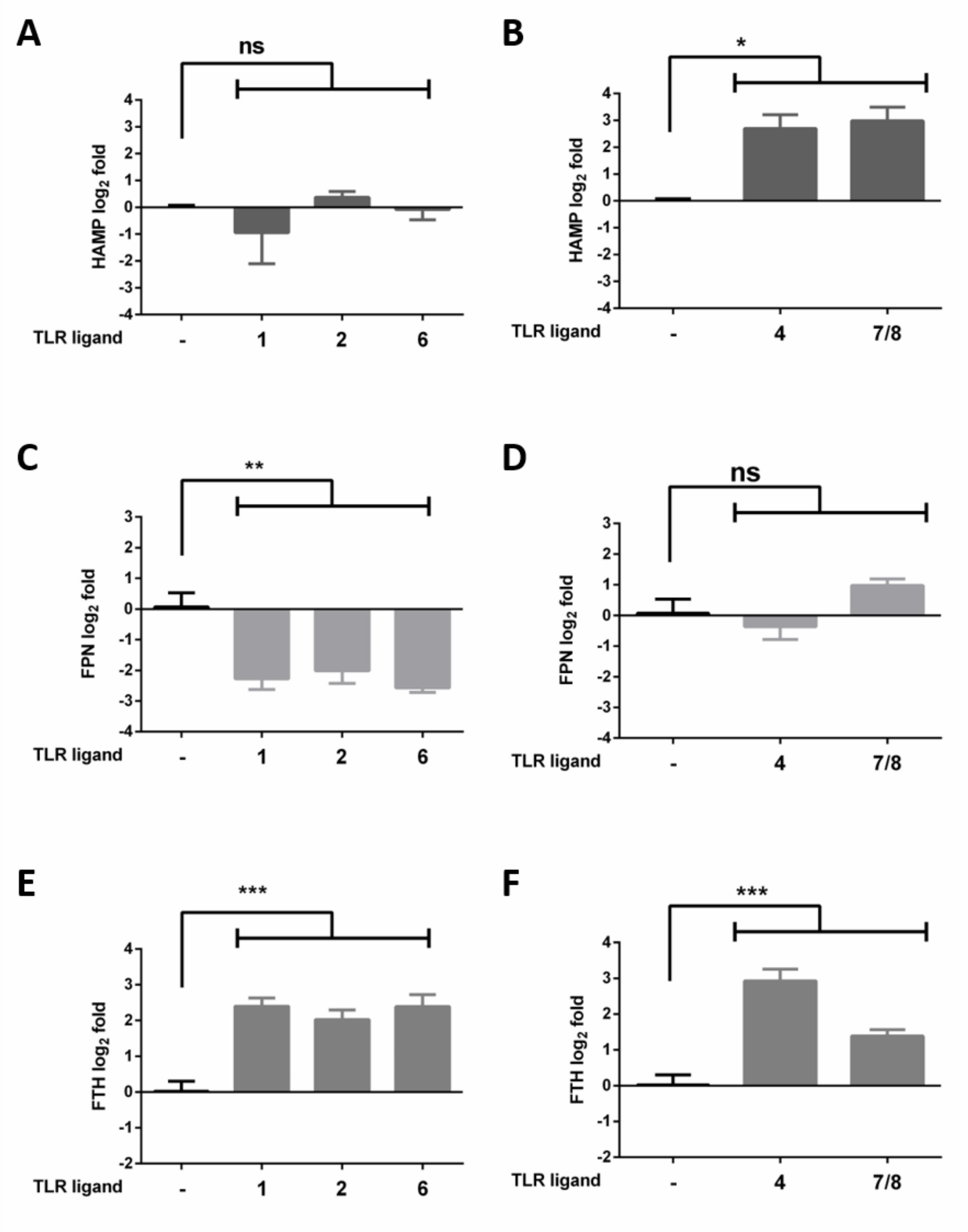
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Fig 4.5: TLR signaling induces hypoferremia through two independent pathways. (A and B) Hepcidin expression in THP-1 macrophages treated with different TLR ligands and organized to show hepcidin non-inducers (A) and hepcidin inducers (B). (C and D) Ferroportin expression in THP-1 macrophages treated with TLR ligands and organized as in A and B. (E and F) Ferritin expression in THP-1 macrophages treated with different TLR ligands organized as in A and B. *$p<0.05$, **$p<0.01$ ***$p<0.001$. All data were from 3 independent experiments.

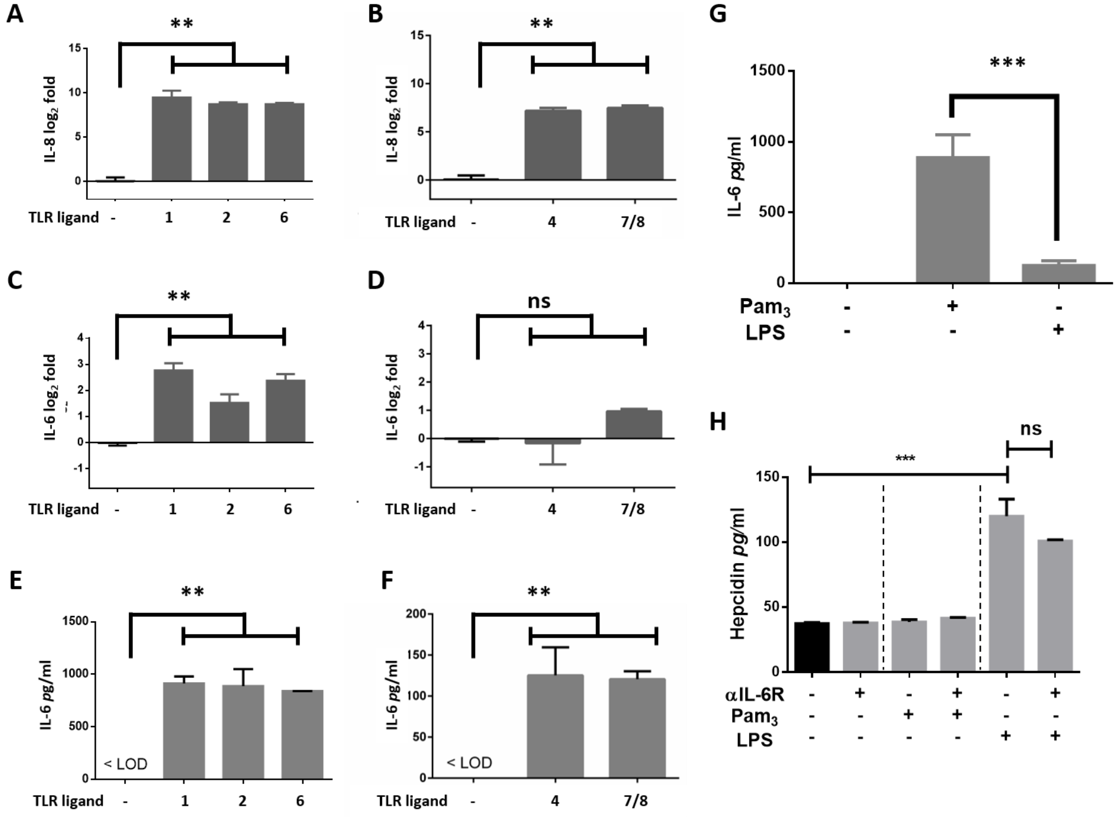

Fig 4.6: TLR-mediated hepcidin induction is independent of IL-6 signaling. (A and B) IL-8 expression in TLR-treated macrophages and organized to show hepcidin non-inducers (A) and hepcidin inducers (B). (C and D) IL-6 expression in TLR-treated macrophages and organized as in A and B. (E and F) IL-6 secretion in TLR treated macrophages and organized as in A and B. (G) IL-6 secretion in macrophages treated with Pam3CSK4 or LPS for 24 hours. (H) Hepcidin secretion in macrophages treated

with Pam3CSK4 or LPS for 24 hours in presence of 1μg/ml IL-6 receptor blocking antibody. **p<0.01, ***p<0.001. All data were from 3 independent experiments.

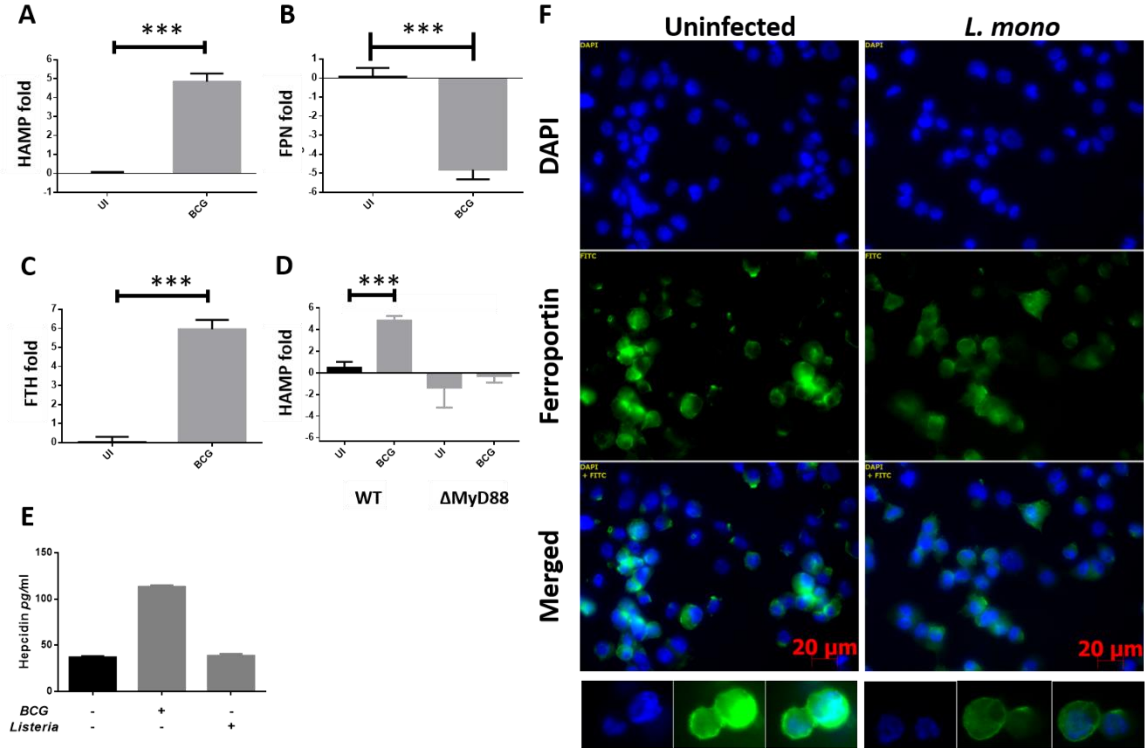

Fig 4.7: BCG induces iron sequestration through hepcidin induction and ferroportin downregulation. (A-C) Transcriptional changes of hepcidin (A), ferroportin (B) and ferritin (C) expression in BCG infected THP-1 macrophages differentiated with 50 nM PMA for 24 hours. (D) Hepcidin mRNA levels of BCG-infected wild-type (WT) and ΔMyD88 THP-1 macrophages. (E) Hepcidin secretion in BCG- or *L. monocytogenes (L. mono)*-infected THP-1 macrophages differentiated as in A. (F) Ferroportin expression in *L mono*-infected THP-1 macrophages. ***p<0.001. All data were from 3 independent experiments.

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Table S4.1: TLR ligands used in this study and concentrations

TLR	Ligand	Concentration
TLR2/1	Pam3CSK4	1 µg/ml
TLR2	Pam2CSK4	500 ng/ml
TLR3	Poly(I:C)	5 µg/ml
TLR4	LPS-EB UP	500 ng/ml
TLR5	FLA-ST	100 ng/ml
TLR6	FSL1	1 µg/ml
TLR7/8	R848	1 µg/ml
TLR9	ODN2216	500 ng/ml

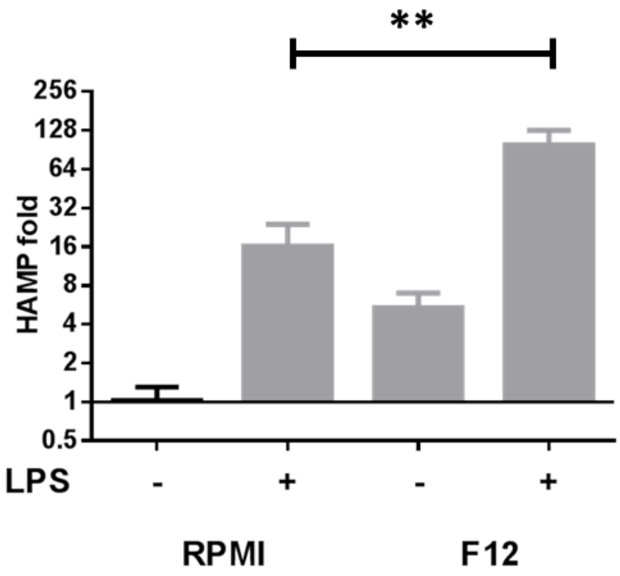

Fig-S4.1: LPS-mediated hepcidin induction is increased in DMEM/F12 medium. THP-1 cells differentiated with 50 nM PMA in DMEM/F12 or C-RPMI as described in Materials and Methods, and stimulated with 500 ng/ml LPS for 24 hours. Data from two independent experiments. Hepcidin expression levels were evaluated by qRT-PCR. $^{**}p<0.01$, $^{***}p<0.001$.

FIG-S2

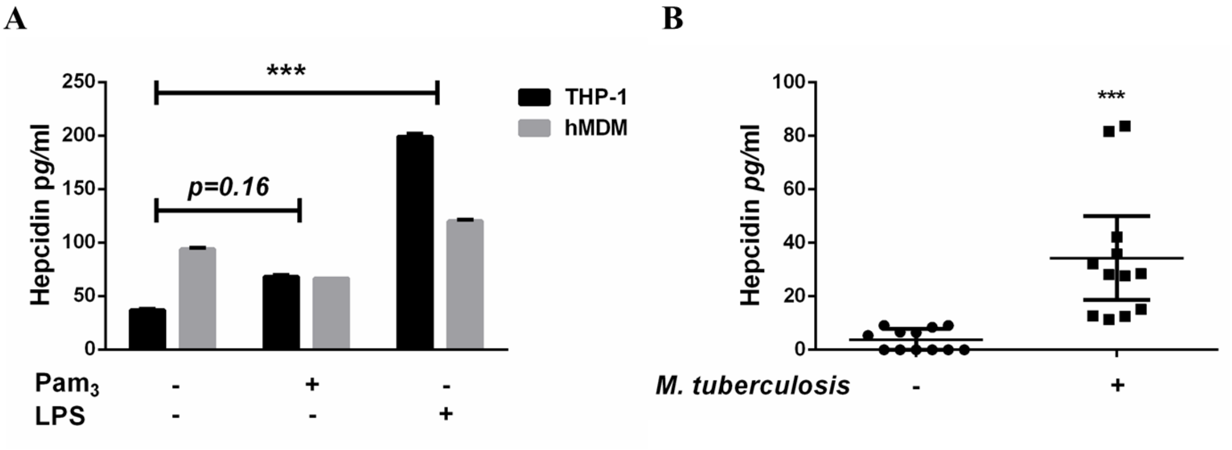

Fig-S4.2: THP-1 mimic pathogen-induced hepcidin expression in human primary macrophages (hMDM). Hepcidin secretion in differentiated THP-1 monocytic cells and primary hMDM 24 hours after LPS (TLR-4 ligand) or Pam3 (TLR-2 ligand) treatment (A) or Mtb infection (B) as described in material and methods section. ***$p<0.001$

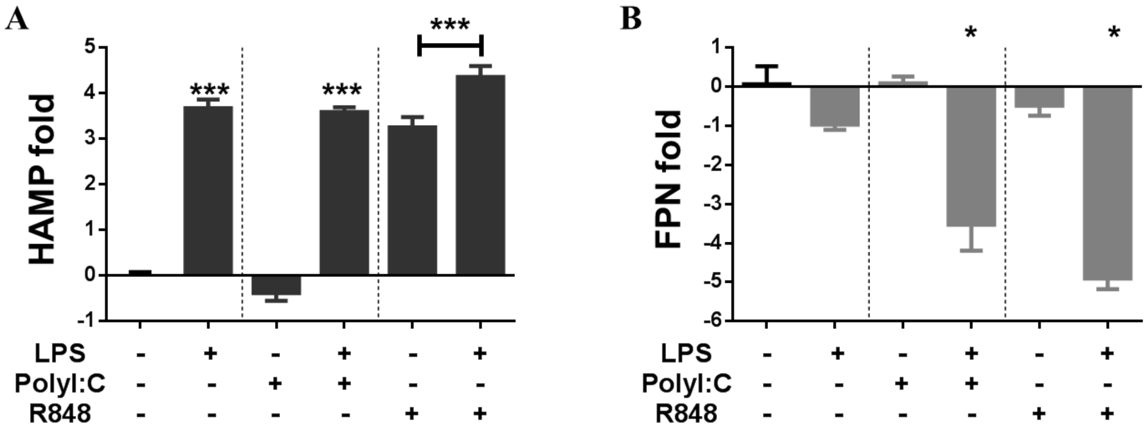

Fig-S4.3: Dual TLR ligand activation significantly downregulates ferroportin expression. Gene transcription levels for hepcidin (A) and ferroportin (B) were assessed by qRT-PCR from differentiated THP-1 cells treated with TLR 3, 4 and 7 ligands. All data were from 3 independent experiments.

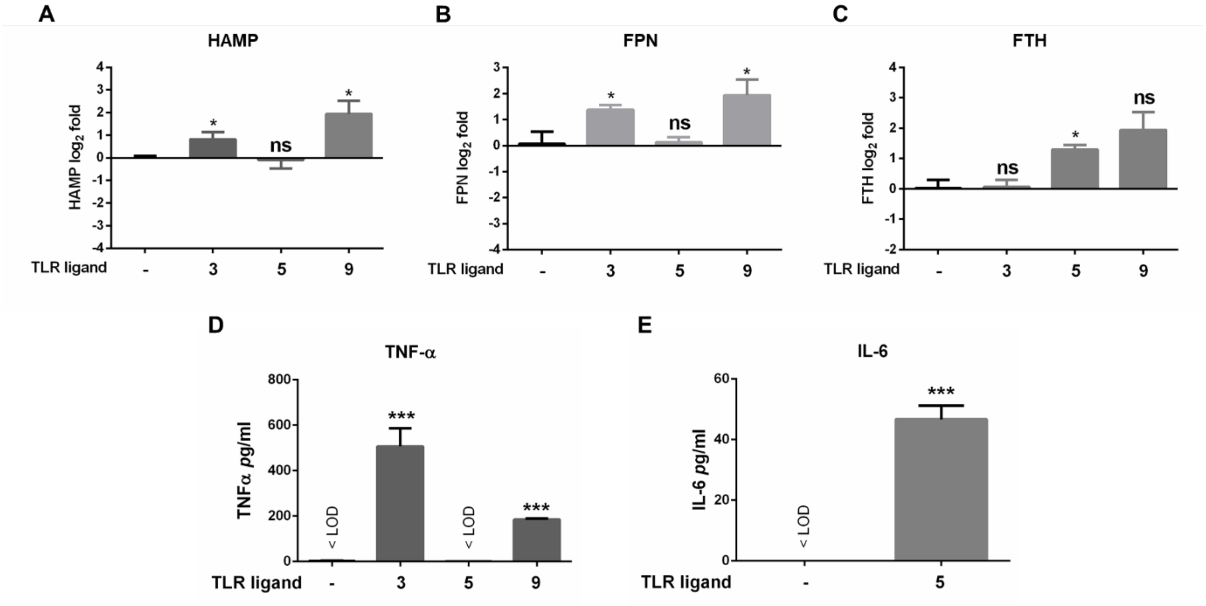

Fig-S4.4: **TLR-3, -5 and -9 activation has no impact on macrophage iron export.** (A-C) Hepcidin (A), Ferroportin (B) and Ferritin (C) expression in differentiated THP-1 cells treated with different TLR ligands. (E and F) TNF-α (E) and IL-6 (F) secretion in TLR treated macrophages **p*<0.05, ***p*<0.01 ****p*<0.001. All data were from 3 independent experiments.

CHAPTER 5

ER-STRESS INDUCES HEPCIDIN IN HUMAN MYELOID CELLS

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[1] Abreu R, Quinn F., Giri P. To be submitted as a letter to the editor of Blood.

Letter to the Editor

Iron is a crucial micronutrient with an important role in multiple biological processes, however, in excess can lead to severe toxicity through reactive oxygen species (ROS) production by the Fenton reaction. Iron dysregulation associated-disorders, such as anemia and hemochromatosis afflict more than 800 million people worldwide and one in every 200 people of northern European descent[1], respectively. Hereditary hemochromatosis (iron overload) results from deficient hepcidin expression or function and leads to relevant health complications such as chronic fatigue, joint pain, spleno- or hepatomegaly and in more severe cases can result in incurable metabolic disorders, *e.g.* diabetes, cirrhosis, or liver cancer[2]. Anemia of chronic diseases (ACD) accounts for more than 40% of total anemia cases worldwide, frequently results in decreased life quality due to fatigue, dizziness, and impaired cognitive functions and, unlike iron deficiency, anemia is not resolved through iron supplementation therapy[3]. ACD results from increased hepcidin expression and secretion leading to decreased intestinal iron absorption and increased iron accumulation in iron storage cells such as hepatocytes and macrophages[3]. Furthermore, anemia correlates with increased risk of acute coronary syndrome, myocardial infarction and long-term obesity[3]. Here we explore the mechanism regulating hepcidin expression in myeloid cells, unveiling endoplasmic-reticulum stress as the major inducer of this peptide in macrophages.

Hepatic hepcidin expression and regulation is well described during dietary iron overload or inflammatory conditions[4]. During iron overload, hepcidin expression is centrally dependent on bone morphogenic protein (BMP) 6 signaling, which is up-regulated by increased intracellular iron levels in hepatocytes[5]. BMP receptor activation phosphorylates and initiates Sma- and Mad-related protein (SMAD) signaling leading to direct hepcidin transcriptional up-regulation[6,7]. Increased extracellular iron levels and consequent transferrin (Tf) saturation is sensed by the Tf receptors, hemojuvelin and hemochromatosis protein, increasing BMP6/BMP receptor signaling[8]. Aside from hepatocytes, macrophages also express and secrete hepcidin in response to inflammatory stimuli[9–11] and are thought to have a significant impact in the regulation of local iron levels during early stages of infection. The hepcidin regulatory mechanisms in myeloid cells remain elusive, but are strictly dependent on inflammatory stimuli[12]. In human macrophages TLR4 signaling strongly up-regulates hepcidin expression[13–15]. In contrast, ferric ammonium citrate supplementation alone has no impact on hepcidin expression, but, in combination with inflammatory stimuli, significantly enhances TLR4-mediated hepcidin expression (Abreu, chapter 4). To test the role of BMP6 signaling in TLR4-mediated hepcidin expression in iron supplemented medium we quantified BMP6 transcriptional expression in THP-1 macrophages 24 hours after LPS treatment by qRT-PCR. LPS significantly inhibits BMP6 expression in macrophages (Fig 1A) indicating that LPS-mediated hepcidin expression in macrophages is independent of BMP6 signaling. Furthermore, BMP6 is undetectable in media supernatants of THP-1 macrophages (data not shown) and SMAD1/5/8 phosphorylation is decreased after LPS treatment (Fig 1B), further supporting the hypothesis that hepcidin expression in macrophages is independent of BMP6 signaling. TGF-β1 has been recently reported to activate SMAD1/5 signaling in human macrophages through TGF-β receptor 1 (ALK5) leading to hepcidin transcriptional upregulation[16]. In our lab TGF-β treatment in THP-1 macrophages has showed no impact on hepcidin secretion levels in media supernatants

(Fig 2A), which together with the abovementioned decrease in phosphorylated SMAD1/5/8 after TLR-treatment, leads us to conclude that TLR-induced hepcidin expression in macrophages is independent of BMP6, TGF-β or SMAD signaling.

During inflammation, IL-6 signaling is the major hepcidin inducer in hepatocytes. IL-6 receptor activation and phosphorylated STAT3 directly up-regulate hepcidin transcription in hepatocytes and are required for hepatic hepcidin secretion during acute infection *in vivo*[17,18]. TLR signaling also has been shown to significantly up-regulate hepcidin expression in hepatocytes and leukocytes, hypothetically through increased IL-6 secretion[15]. Nonetheless, we recently observed that TLR2 signaling does not induce hepcidin expression despite abundant IL-6 secretion in THP-1 macrophages (Abreu, chapter 4). Similarly, when we infected THP-1 macrophages with *Listeria monocytogenes*, which prominently activates TLR2 signaling, we observed no significant changes on hepcidin secretion (Fig 1C and D). Contrastingly, LPS treatment or *Salmonella enterica* serovar typhimurium infection significantly increases hepcidin secretion in human THP-1 macrophages (Fig 1C), regardless of the significantly decreased IL-6 levels in the culture supernatants (Fig1 D). Furthermore, LPS-treated macrophages show decreased total STAT3 levels (Fig 1B) and phosphorylated STAT3 could not be detected in THP-1 macrophages after any of the TLR treatments or infections reported in this study besides direct IL-6 stimulation (10 ng/ml) (Fig 1E). Despite a significant increase in hepcidin secretion after IL-6 treatment (10 ng/ml), IL-6 receptor blocking (1 μg/ml) has no impact on LPS-mediated hepcidin in THP-1 macrophages supporting the hypothesis that in these cells LPS-induced hepcidin expression is independent of IL-6 signaling (Fig 2A and B). Considering the IL-6 physiological levels in healthy (10-30 pg/ml) and chronically inflamed patients (<500 pg/ml)[19,20] and those detected in the supernatants of TLR stimulated THP-1 macrophages (<1 ng/ml) we suspect that the marginal increase in hepcidin secretion after IL-6 treatment (10 ng/ml) is an artifact of excessive IL-6 signaling and irrelevant under physiological settings.

Other mechanisms have been reported as alternative hepcidin inducers during infection. Although not recapitulated *in vivo*, IL-1β induces hepcidin expression in hepatocytes *in vitro* hypothetically through activation of NF-κB transcription factor[13]. Bacterial infection and TLR signaling prominently activate NF-κB, thus we assessed the impact of NF-κB irreversible specific inhibitor (Bay 11-7082) in LPS-mediated hepcidin induction. THP-1 macrophages pre-treated with 1 μM Bay 11-7082 for one hour have slightly decreased hepcidin secretion compared to untreated controls (*p*=0.038), although NF-κB inhibition has no impact on hepcidin secretion levels after LPS treatment (*p*=0.38), proving that TLR-mediated hepcidin induction is independent of NF-κB signaling (Fig 2B). Similarly, IL-1β treatment, which strongly activates NF-κB, has no impact on hepcidin secretion in THP-1 macrophages further supporting this hypothesis (Fig 2A).

Endoplasmic-reticulum (ER) stress increases iron sequestration in the liver and spleen *in vivo* through hepcidin upregulation. In hepatocytes ER-stress mediated CREBH (cyclic AMP response element binding protein H) activation leads to direct hepcidin transcriptional upregulation, but a similar impact on myeloid cells has not been assessed[21,22]. Bacterial infection and TLR4 signaling rapidly induce ER-stress in macrophages[23] (Fig 2C), thus we evaluated the impact of tunicamycin, a strong ER-stress inducer, in hepcidin secretion by THP-1 macrophages. After five hours treatment with 5 µg/ml tunicamycin, THP-1 macrophages secrete 20 times more hepcidin than untreated controls and 10 times more than IL-6 stimulated cells (Fig 2A). This effect could be inhibited by treatment with the ER-stress specific inhibitor 4-phenylbutyrate (PBA) (Fig 2D), indicating that ER-stress is the primary inducer of hepcidin expression in macrophages. To assess if LPS-induced ER-stress was responsible for TLR-mediated hepcidin expression, we treated THP-1 macrophages with LPS in the presence of the ER-stress inhibitor PBA and measured hepcidin secretion in the supernatants. As expected, PBA-treated macrophages have decreased hepcidin secretion (p<0.001) and are unresponsive to LPS treatment showing no significant changes in hepcidin secretion 24 hours after LPS stimulation (Fig 2B and D). Similarly, *S. typhimurium* infection in PBA-treated macrophages does not produce a significant change in hepcidin secretion compared to uninfected controls, further supporting the hypothesis that infection-mediated hepcidin secretion in macrophages is dependent on TLR-induced ER-stress. In the future, we shall assess if ER-stress hepcidin induction in macrophages is related to CREBH activation as previously reported in hepatocytes[21], and the relevance of this mechanism on intracellular iron sequestration in tissue macrophages during early stages of infection.

Altogether, this study comprehensively examines, for the first time, the mechanisms involved in hepcidin induction in hepatocytes and assesses their role in myeloid cells. Here we show that BMP-6 and IL-6, which are generally recognized as the major players in hepatic hepcidin induction, are not responsible for bacterial-induced hepcidin expression in macrophages during infection. Furthermore we unveil TLR-induced ER-stress as the primary mechanism leading to increased hepcidin secretion in human myeloid cells. The outcome of this study greatly impacts the current perspective on iron regulation during infection in myeloid cells, and exposes ER-stress as an important therapeutic target for the treatment of ACD.

Fig 1

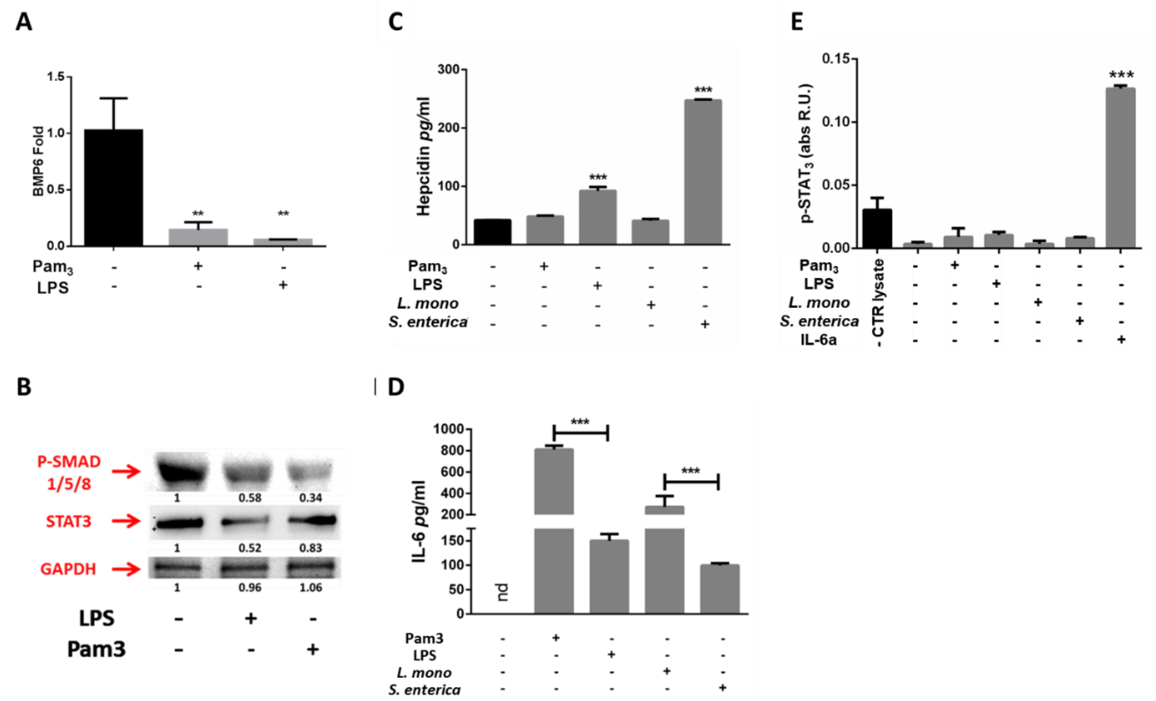

Fig 5.1: **BMP6 and IL-6 does not induce hepcidin in human macrophages.** A) Hepcidin transcriptional changes in THP-1 macrophages after TLR4 stimulation (LPS 500 ng/ml) in iron supplemented media (100 μM FeAC) measured by qRT-PCR. B) Hepcidin secretion in media supernatants of THP-1 macrophages 24 hours after TLR2 (Pam3), TLR4 (LPS) stimulation, or *L. monocytogenes* and *S. enterica* infection. C) BMP6 transcriptional changes after TLR2 (Pam3) or TLR4 (LPS) stimulation measured by qRT-PCR. D) IL-6 secretion in media supernatants of THP-1 macrophages 24 hours after TLR2 (Pam3), TLR4 (LPS) stimulation, or *L. monocytogenes* and *S. enterica* infection. E) SMAD1/5/8 phosphorylation levels and total STAT3 levels in THP-1 macrophages after TLR2 (Pam3) or TLR4 (LPS) stimulation. A, C, D and E are data from three independent experiments analyzed by two-sided *t*-test. ***$p<0.001$. B is representative of two independent experiments.

Fig 2

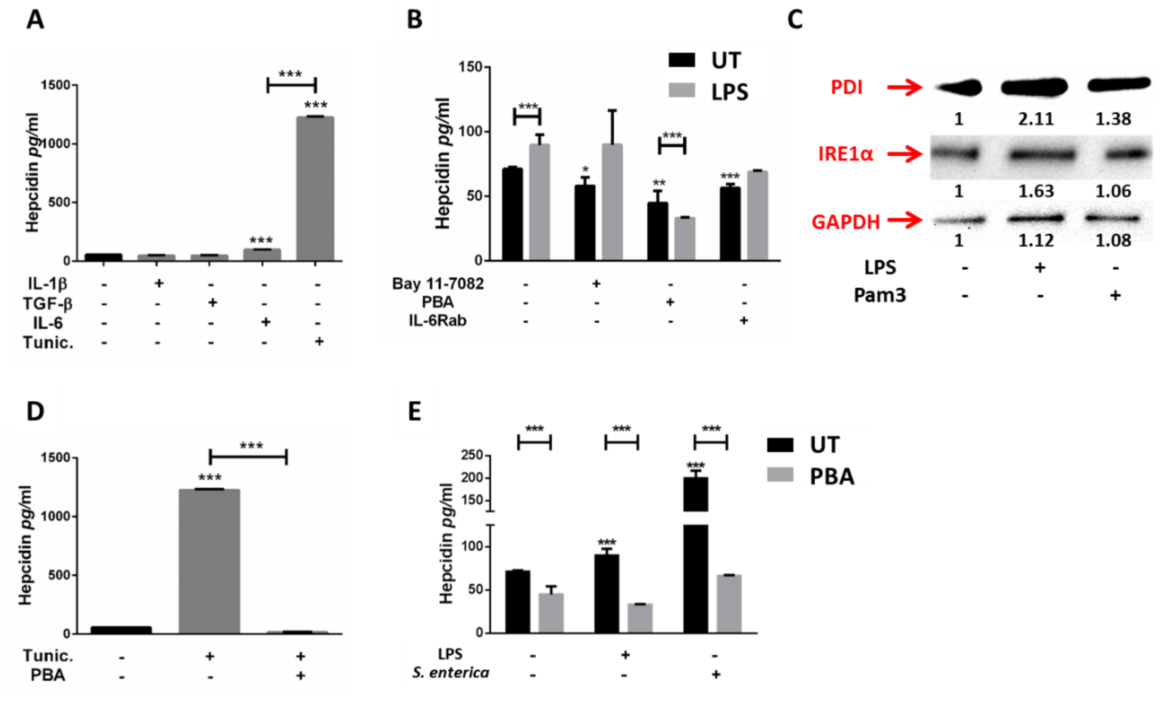

Fig 5.2: **ER-stress is the major regulator of hepcidin expression in human macrophages.** A) Hepcidin secretion in media supernatants of THP-1 macrophages 24 hours after stimulation with hepcidin-inducing cytokines IL-1β, TGF-β, IL-6 or 6 hours after ER-stress inducer tunicamycin (Tunic.). B) Hepcidin secretion in media supernatants of tunicamycin-treated THP-1 macrophages pre-treated with ER-stress inhibitor 4-phenylbutyrate (PBA). C) Hepcidin secretion in media supernatants of LPS-stimulated THP-1 macrophages, pre-treated with NF-κB inhibitor (Bay 11-7082), ER-stress inhibitor (PBA) or treated in presence IL-6 receptor blocking antibody (IL-6Rab). E) Expression of ER-stress markers protein disulfide isomerase (PDI) and inositol-requiring enzyme 1 alpha (IRE1α) in THP-1 macrophages after TLR2 (Pam3) or TLR4 (LPS) stimulation. A, B, D and E are data from three independent experiments analyzed by two-sided *t*-test. ****p*<0.001. C is representative of two independent experiments.

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CHAPTER 6

INTERFERON-γ PROMOTES IRON EXPORT IN HUMAN MACROPHAGES TO LIMIT INTRACELLULAR BACTERIAL REPLICATION

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[1] Abreu R, Quinn F., Giri P. In submission to Scientific Reports, April 13th 2018

Abstract

Salmonelliosis and Listeriosis together accounted for more than one third of foodborne illnesses in the United States and almost half the hospitalizations for gastrointestinal diseases in 2015. Tuberculosis afflicted over 10 million people worldwide causing almost 2 million casualties in 2015. Regardless of its intrinsic difference between *Listeria monocytogenes*, *Salmonella enterica* and *Mycobacterium tuberculosis*, these pathogens share the ability to survive and persist inside the macrophage and thrive in hemochromatosis patients. IFNγ is a central cytokine in host defense against intracellular pathogens and has been shown to promote iron export in macrophages. Here we hypothesize that IFNγ decreases iron availability to intracellular pathogens consequently limiting replication in macrophages. In this study we show that IFNγ regulates the expression of iron-related proteins hepcidin, ferroportin and ferritin to induce iron export from macrophages. *L. monocytogenes, S. enterica* and *M. bovis* BCG infection significantly induce iron sequestration in human macrophages. In contrast, IFNγ significantly reduces hepcidin secretion in *S. enterica* or BCG infected macrophage. Similarly, IFNγ-activated macrophages express higher ferroportin levels than untreated controls even after infection with *Listeria monocytogenes*, which greatly downregulate ferroportin expression. In a nutshell, IFNγ significantly inhibits pathogen-associated intracellular iron sequestration in macrophages consequently decreasing iron availability to intracellular bacterial pathogens.

Introduction

The current HIV/AIDS global epidemic with an increased elder population in Europe and the USA requires a new awareness to self-resolving diseases and opportunistic infections. Between Europe and the USA more than 3 million people live with HIV(ECDC/WHO 2016; CDC 2016) and 15 to 19% of the population is over 65-years old (Eurostats, USA census bureau), estimating 50 million people with weakened immune systems and increased risk of serious complications upon infection with self-resolving pathogens such as *Listeria monocytogenes* (*L. mono)* or *Salmonella enterica (S. enterica)* (USDA/FDA 2011). Salmonellosis accounts for 38% of all foodborne diseases in the United States of America and the second most commonly reported gastrointestinal infection in Europe (ECDC 2016; CDC 2016). Listeriosis reports are less common but have the highest rates of hospitalization and death among all foodborne illness (ECDC 2016). Tuberculosis is the leading cause of death in HIV infected people and one of the deadliest diseases in the world on its own (CDC; ECDC; WHO). In Europe, almost 60 000 new cases of tuberculosis were reported in 2014 (ECDC 2016), while in the USA almost 10 000 people were afflicted by this disease during 2015 (CDC 2015).

Despite the intrinsic differences between *L. monocytogenes*, *S. enterica* and *Mycobacterium tuberculosis (M. tuberculosis)*, these pathogens share the ability to survive and replicate inside the macrophages (Monack et al. 2004). By inhibiting macrophage antimicrobial functions, these pathogens evade both innate and adaptive immune responses and persist within the host for long periods of time (Kaufmann 1993). Furthermore all these three pathogens are associated with reactivation and recurrent infection in immunocompromised individuals such as elder or HIV infected patients

(Decker et al. 1991; Kales & Holzman 1990; Hung et al. 2007; Levine & Farag 2011; CDC 2016).

IFNγ is a critical cytokine during innate and adaptive immune responses against intracellular bacteria (Weiss & Schaible 2015; Flannagan et al. 2009). IFNγ knock-out mice are severely susceptible to *Listeria* (Harty & Bevant 1995), *Salmonella* (Bao et al. 2000) and *Mycobacteria* (Kawakami et al. 2004) infection. In humans, impaired IFNγ signaling is associated with increased risk of tuberculosis (Bellamy 2003). During the adaptive immune response IFNγ controls the differentiation of $CD4_{Th1}$ effectors T cells, which mediate cellular immunity against intracellular bacterial infections. IFNγ-activated macrophages have upregulated antigen presentation, increased phagocytosis and proinflammatory cytokines secretion, and enhanced production of superoxide radicals, nitric oxide, and hydrogen peroxide (Liu & Modlin 2008; Kaufmann 1993). Recently IFNγ has also been shown to increase ferroportin expression in *S. enterica* infected murine macrophages, promoting iron export and limiting bacterial replication (Nairz et al. 2008).

Aside from the ability to survive and persist inside macrophages, *L. monocytogenes, S. enterica* and *M. tuberculosis* also share the ability to thrive in iron rich environments (Nugent et al. 2015; Haschka et al. 2015; Gomes et al. 1999; Siegrist et al. 2009). Knock-out of iron acquisition genes in siderophilic bacteria results in severely attenuated strains (Siegrist et al. 2009; De Voss et al. 2000; Siegrist et al. 2009), while host iron dysregulation, like in hemochromatosis patients, is greatly associated with worsened disease outcomes with any of the herein reported pathogens (Khan et al. 2007).

Here we show that IFNγ promotes iron export and efficiently prevents pathogen-associated intracellular sequestration in human macrophages during infections with *L. monocytogenes, S. enterica* and the *M. tuberculosis* BSL2-model, *M. bovis* BCG (BCG). Furthermore, the resultant decrease in intracellular iron availability to these siderophilic bacteria significantly limits bacterial replication inside the macrophage resembling the effect of iron chelation therapy. The outcome of this work unravels a novel mechanism by which IFNγ limits intracellular bacteria replication in human macrophages.

Results

IFNγ favors iron export in human macrophages

IFNγ has been previously shown to decrease intracellular iron levels and limit *Salmonella* replication in mouse macrophages (Nairz et al. 2009). To assess if IFNγ can also modulate iron related genes in human macrophages we treated human THP-1 macrophages with human recombinant IFNγ (200 U/ml) and quantified transcriptional expression of the iron regulator hepcidin, iron exporter ferroportin and intracellular iron storage protein ferritin by qRT-PCR. In agreement with the abovementioned study, the ferroportin transcriptional levels are 2.5-fold higher (±0.23, p=0.005) 16h after IFNγ treatment compared to untreated controls (Fig 1A). On the other hand, transcriptional expression of the ferroportin downregulator hepcidin is decreased by approximately 70% (p<0.001) after IFNγ treatment (Fig 1B), again biasing towards and iron export phenotype. This transcriptional data is further supported by respective protein levels (Fig 1D and E). Hepcidin secretion to the medium supernatants is significantly decreased (Fig 1D, p<0.001) after IFNγ treatment while surface ferroportin is increased (Fig 1E).

Interestingly, despite the difference in ferroportin and hepcidin expression levels, IFNγ treatment does not alter expression of the iron storage protein ferritin (Fig 1C).

Siderophilic bacteria manipulate host iron-related proteins to favor intracellular iron sequestration

Intracellular siderophilic bacteria such as *M. tuberculosis* (Mtb) or the BSL2 model *M. bovis* BCG (BCG), *L. mono.* or *S. enterica* prominently activate Toll-like receptor signaling (Weiss & Schaible 2015). Interaction and activation of Toll-like receptors expressed by macrophages induces intracellular iron sequestration both through increased hepcidin secretion and decreased ferroportin expression (Abreu, chapter 4). To test if these siderophilic pathogens can manipulate host iron-related proteins in the macrophage, we infected THP-1 macrophages with Mtb, BCG, *L mono*, *S. enterica*, and quantified hepcidin secretion by ELISA at the peak of infection. Upon infection, both Mtb and BCG infected macrophages secrete significantly more hepcidin than respective uninfected controls, 48h and 24h after infection respectively (mean dif. 88.6±2.8 pg/ml and 76.2±1.2 pg/ml respectively, $p<0.0001$). This represents an approximate 3-fold increase. In the same way, infection with a Gram-negative intracellular pathogen, *S. enterica*, also results in increased hepcidin secretion, in agreement with our previous report that suggests TLR4 activation is responsible for hepcidin expression in macrophages during infection (Fig 2A).

On the other hand, *L. mono* infection has no impact on hepcidin secretion (Fig 2A), although leads to direct ferroportin downregulation, independent of hepcidin expression (Fig 2B and C). *L. mono* infected macrophages express lower levels of surface ferroportin 6h post infection compared to uninfected controls (Fig 2B), a 60% decrease measured by mean fluorescence intensity. To confirm that *L. mono* downregulates ferroportin through a hepcidin-independent mechanism, we silenced hepcidin expression through hepcidin gene specific lentiviral ShRNA (Fig 2C and 5A). Scramble negative controls (ShScram) and ShRNA hepcidin KD (ShHAMP) THP-1 macrophages infected with *Listeria* show similar surface ferroportin levels (Fig S1) supporting the hypothesis that this pathogen can promote intracellular iron sequestration, through direct ferroportin downregulation.

Pathogen-associated intracellular iron sequestration promotes intracellular replication

Intracellular pathogens modulate macrophage iron-related proteins that favor iron sequestration (Fig 2), so we next tested if BCG, *L. mono* or *S. enterica* infected macrophages have increased iron content compared to uninfected controls by Prussian blue iron staining. Uninfected macrophages have low iron retention with minimal iron staining (Fig 3A). However, upon infection with any of the abovementioned siderophilic bacteria, macrophages have increased intracellular iron levels as observed by increased blue granules (Fig 3B). Interestingly, BCG, *L. mono* and *S. enterica* result in very different iron staining patterns; *L. mono* infected macrophages have increased intracellular iron levels but with a similar pattern of uninfected cells (small blue granules dispersed in the cytoplasm), BCG induces increased iron retention in the nucleus (overlaps with nuclear red staining) with some diffuse blue iron staining in the cytoplasm , and finally S. *enterica* infected macrophages show large iron stained granules in the cytoplasm or intracellular vesicles (Fig 3A).

Iron chelation decreases replication of intracellular pathogens

Iron dysregulation is associated with worsen disease prognosis upon infection with siderophilic bacteria such as Mtb, BCG, *L. mono* and *S. enterica*. In the opposite way, iron chelation has proven to be an effective therapy *in vitro* and *in vivo* against some of these siderophilic pathogens (Kontoghiorghes et al. 2010). To evaluate if increased intracellular iron sequestration was essential for bacterial replication, we infected THP-1 macrophages with Mtb, *L mono* and *S. enterica* in presence of the iron chelators deferoxamine or deferiprone and measured intracellular replication through gentamicin protection assay. As expected iron chelation significantly decreases intracellular replication of the siderophilic bacteria Mtb (90% less 72 hours post-infection, Fig3B), *L mono* (72% less 8 hours post-infection, Fig 3C) and *S. enterica* (89% less 16 hours post-infection, Fig 3D).

IFNγ prevents pathogen associated iron modulation in macrophages

IFNγ treatment and intracellular pathogens have opposing polarizing effects on macrophages (Fig 1 and 2), so we next tested if IFNγ treatment can prevent iron retention in macrophages infected with BCG, *L. mono* or *S. enterica*. THP-1 macrophages were activated with 50 U/ml of human recombinant IFNγ overnight and infected with different intracellular pathogens. At peak of infection we assessed ferroportin levels by immunofluorescence and hepcidin secretion by ELISA. Similar to what we observed with uninfected macrophages (Fig 1), IFNγ treatment increases ferroportin expression in THP-1 macrophages infected with *L. mono* (upper panel), BCG (middle panel), S. *enterica* (bottom panel of Fig 4A). MFI quantification reveals that IFNγ treatment significantly increases ferroportin expression by 60 and 74% for *L. mono* and *S. enterica* respectively (Fig S2).

Additionally, IFNγ also decreases hepcidin secretion in the media supernatants of *L. mono* (mean dif. 37.9±1.5 pg) and BCG (mean dif. 50.9±1.5 pg) infected macrophages at 8 and 24 hours post-infection, respectively (Fig 4D and E). Surprisingly, IFNγ only marginally inhibits hepcidin secretion from *S. enterica* (mean dif. 47.2±1.97 pg, $p<0.001$) infected macrophages to levels still significantly higher than uninfected controls (Fig 4F).

IFNγ limits intracellular *S. enterica* replication in macrophages through hepcidin inhibition

IFNγ treatment counteracts pathogens modulation of iron-related genes (Fig 4) favoring iron export. IFNγ has been previously reported to limit iron availability to intracellular pathogens through upregulation of ferroportin leading to decreased bacterial replication. To assess if hepcidin inhibition would translate into increased ferroportin expression and decreased intracellular bacterial replication, we silenced hepcidin expression through hepcidin gene specific lentiviral ShRNA (ShHAMP) (Fig 5A) in THP-1 macrophages and measured intracellular replication in a gentamicin protection assay. Just like IFNγ treatment, hepcidin silencing leads to increased ferroportin expression in uninfected macrophages favoring iron export (Fig 5B). Upon infection with *S. enterica* ShHAMP THP-1 macrophages have significantly decreased intracellular bacterial replication than respective negative scramble controls (ShScram) (90% decrease 16 hours post-infection) at peak of infection (16 hours post infection) (Fig 5C). In contrast, *L. mono* replication is altered in ShHAMP THP-1 macrophages, suggesting that *Listeria*-mediated iron sequestration is hepcidin-independent (Fig 5D).

IFNγ limits intracellular bacterial replication in macrophages through activation of multiple anti-microbial mechanisms (Boehm et al. 1997). To confirm that the concentrations tested in this work inducing iron export also reduce intracellular bacterial replication, we treated THP-1 macrophages with 50 U/ml IFNγ overnight, infected with *L. mono* and *S. enterica* and quantified intracellular bacterial burden in a gentamicin protection assay. Both *L. mono* and S. *enterica* infected macrophages have significantly decreased intracellular bacterial burden after IFNγ treatment at peak of infection. IFNγ treatment results in an 80% decrease in *L. mono* intracellular burden 6 hours post-infection. In *Salmonella*-infected macrophages, IFNγ has a significant impact in intracellular CFU 16 hours post-infection, where it translates into a 90% decreased in CFU counts compared to untreated controls (Fig 5E and F).

Discussion

The host immune response to intracellular bacteria is a complex network of pro- and anti-inflammatory mediators assuring efficient bacterial killing with minimal tissue damage (Kaufmann 1993). In contrast, bacterial persistence within the host is a fine tune of virulence factors that allow bacterial survival within the host with minimal activation of the surveilling immune system (Monack et al. 2004). *M. tuberculosis*, *L. monocytogenes* and *S. enterica* serovar typhimurium are three intracellular bacteria that persist within the macrophage and efficiently avoid the host immune system. Despite the intrinsic different mechanism and virulence factors involved in bacterial survival and replication inside the macrophage, these three pathogens share the ability to avoid or inhibit macrophage anti-microbial functions, e.g. phagosome maturation, phagolysosome fusion or induction of nitrogen and oxygen reactive species (Monack et al. 2004). In contrary, IFNγ macrophage activation promotes bacterial killing through direct induction of the abovementioned antimicrobial mechanisms (Schoenborn & Wilson 2007). Here we report a novel mechanism by which IFNγ limits intracellular bacteria replication in macrophages. In human macrophages IFNγ promotes iron export and efficiently prevents pathogen-associated intracellular sequestration. The consequent decrease in intracellular iron availability to these siderophilic pathogens significantly limits bacterial replication inside the macrophage.

Iron is a crucial micronutrient to all forms of life with important biological functions as a component of molecule sensing, transporting and storing oxygen, of enzymes involved in oxidation and reduction of substrates during energy production, intermediate metabolism, and the generation of reactive oxygen or nitrogen species for host defense. During infection with siderophilic bacteria, iron chelation greatly decreases intracellular bacterial replication (Cassat & Skaar 2013 and Fig 2). Pathogen-associated intracellular iron sequestration in macrophages is dependent on TLR signaling and mediated through two independent and redundant mechanisms (Abreu, chapter 4). While TLR4 and TLR7/8 signaling induces hepcidin secretion, TLR1, -2 and -6 activation significantly inhibits ferroportin expression in THP-1 human macrophages (Abreu, chapter 4). Consistent with its predominant TLR2 activation (Pamer 2004), *L. monocytogenes* significantly decreases ferroportin expression through a hepcidin-independent mechanism (Moreira et al. 2017 and Fig 2). However, IFNγ treatment significantly increases ferroportin expression in THP-1 macrophages even after *L. monocytogenes* infection (Fig 3A) inhibiting *Listeria*-associated intracellular iron sequestration.

Increased hepcidin expression has been shown to promote intracellular BCG replication (Abreu, chapter 4 and Fig S4), and HIV replication is augmented in hepcidin-treated macrophages (Xu et al. 2010). Gram-negative *S. enterica* significantly induces hepcidin expression (Fig 2), whereas Mtb and BCG, which activate both TLR2 and TLR4 signaling, promote intracellular iron sequestration through hepcidin-dependent and independent mechanisms (Fig 3). In this study we observed that IFNγ inhibits hepcidin secretion in human macrophages (Fig 1) and significantly decreases pathogen-induced hepcidin secretion during BCG or S. *enterica* infection (Fig 4), greatly reducing intracellular iron sequestration in infected macrophages.

Ferroportin overexpression in murine macrophages severely impairs S. *enterica* growth (Chlosta et al. 2006). Similarly, hepcidin knock down through ShRNA HAMP gene silencing, drastically reduces *S. enterica* replication showing that IFNγ-mediated hepcidin-downregulation alone can significantly impact intracellular replication. Furthermore, sodium phenylbutyrate, a strong hepcidin inhibitor in macrophages (Abreu, chapter 5) has been shown to significantly inhibit S. *enterica* growth *in vivo* (Jellbauer et al. 2016). However, *L. monocytogenes* intracellular bacterial burden remains unaltered in ShRNA-HAMP THP-1 macrophages, pointing to IFNγ-induced ferroportin expression as the driving factor to limit bacterial growth during *Listeria* infection.

Bacteria possess a myriad of mechanisms to scavenge the host iron pool and the three pathogens used in this study have extremely disparate iron scavenging strategies (Sheldon et al. 2016). Mtb siderophores, mycobactin and carboxymycobactin, efficiently recruit and scavenge iron in the phagosome (Sritharan 2016). Carboxymycobactin is the major iron-chelator for both free and protein-bound iron in the macrophage phagosome and cytoplasm (Sritharan 2016; Hameed et al. 2015), while surface mycobactin acts as a membrane chelator and iron-transporter recovering iron from carboxymycobactin and host ferritin (Luo et al. 2005). In macrophages, ferritin mostly localizes to the nucleus with minimal cytoplasmic distribution (Surguladze et al. 2005), and BCG-infected macrophages present increased iron retention in the nucleus with some diffuse iron distribution in the cytoplasm (Fig 3). Iron-loaded ferritin has been previously shown to be efficiently recruited to the phagosome and utilized by *Mycobacterium tuberculosis* (Luo et al. 2005). Future studies may explore the impact of IFNγ in intracellular iron distribution in the macrophage and its accessibility to the bacteria.

S. *enterica* inhibits phagolysosome fusion, persisting and replicating inside the immature phagosome compartment (Buchmeier & Heffron 1991). During infection, efficient control of intra-phagosome iron levels by the phagosomal iron exporter NRAMP is essential to limit bacterial replication (Ong et al. 2006; Nairz et al. 2009). *S. enterica* iron acquisition strategies are very similar to other Gram-negative bacteria and mostly dependent on the ferric siderophores enterochelin and salmochelin (Hantke et al. 2003; Nugent et al. 2015). These siderophores scavenge iron from the host protein transferrin and lactoferrin, which is then transported through bacterial outer-membrane receptors IroN and FepA (Hantke et al. 2003; Parrow et al. 2013). Besides this mechanism S. *enterica* can also utilize heme-iron sources inside the phagosome, although this seems to be more prominent during infection of hemophagocytic macrophages (Parrow et al. 2013). Consistent with the use of intra-phagosomal iron sources, S. *enterica* infected macrophages have localized iron aggregates (Fig 3) possibly associated with immature phagosomes where the bacteria persist. Although iron supplementation decreases bacterial survival during early stages of infection probably through increased ROS generation, at 16 hours post infection increased iron levels are detrimental for the host and facilitate bacterial replication (Fig S3B). This supports the hypothesis of pathogen-mediated iron recruitment and accumulation in the phagosome which counteracts NRAMP iron export from the phagosome at later stages of infection required for efficient bacterial clearance. Aside from conforming iron localization to phagosome, future studies may assess how iron gets recruited to this compartment and how NRAMP impacts iron distribution in the macrophage.

Unlike Mycobacterium and Salmonella, siderophore synthesis genes are absent in *L. monocytogenes* genome and direct iron supplementation has no impact on intracellular bacterial replication in macrophages (Fig S3A). Therefore heme-bound iron is proposed as the major iron source utilized by this pathogen during macrophage infection (Lechowicz & Krawczyk-Balska 2015). Phagosomal activation of the pore-forming protein listeriolysin-O leads to bacterial escape from the phagosome to the cytoplasm (Flannagan et al. 2009; Schnupf & Portnoy 2007). Once in the cytoplasm expression of the ferrochrome ABC transporters *hupCGD (lmo2429/30/31)* enhances iron acquisition from heme-proteins in the cytoplasm (Lechowicz & Krawczyk-Balska 2015; Haschka et al. 2015). The diffuse cytoplasmic distribution of intracellular iron in *Listeria*-infected macrophages (Fig 3) may represent an increase in heme-proteins which can be efficiently be used as an iron source. In the future it would be interesting to identify the major heme-proteins targeted by *L. monocytogenes* for iron scavenging and evaluate the impact of IFNγ signaling in the expression these same proteins.

Hepcidin was first identified as an antimicrobial peptide and its protective impact during infection with extracellular pathogens has been extensively reported (Park et al. 2001; Krause et al. 2000). Just like lactoferrin, hepcidin efficiently decreases extracellular iron availability to pathogens such as *Vibrio cholerae* (Arezes et al. 2015; Ong et al. 2006). However, during infection with intracellular pathogens, hepcidin-mediated intracellular iron sequestration in macrophages is detrimental for the host and facilitates bacterial replication (Xu et al. 2010; Chlosta et al. 2006; Drakesmith et al. 2015; Kasvosve 2013). Furthermore, hepcidin has been reported to play some anti-inflammatory role during chronic infections which could further dampen an effective immune reponse against persistent intracellular pathogens (Nemeth et al. 2003; De Domenico et al. 2012).

IFNγ is a central cytokine for innate and adaptive immunity against intracellular pathogens. IFNγ upregulates both MHCI and II antigen presentation, contributes to macrophage activation by increasing phagocytosis and priming the production of proinflammatory cytokines and potent antimicrobials, including superoxide radicals, nitric oxide, and hydrogen peroxide (Boehm et al. 1997). IFNγ also controls the differentiation of $CD4_{Th1}$ effector T cells, which mediate cellular immunity against intracellular bacterial infections. The role of IFNγ in intracellular iron availability to *Salmonella enterica* has been previously reported. In this report, IFNγ-mediated nitric oxide production significantly increased ferroportin expression in murine macrophages which significantly contributed to limit intracellular bacteria replication (Boehm et al. 1997). Here we describe a similar outcome where IFNγ strongly promotes iron export in human macrophages though increased ferroportin expression and decreased hepcidin secretion. The consequent decrease in intracellular iron availability severely limits replication of three different human pathogens *Listeria monocytogenes, Salmonella enterica* and *Mycobacterium tuberculosis*. This study elucidates a novel mechanism by which IFNγ controls intracellular bacterial replication and exposes iron dysregulation as an important factor during both innate and adaptive immunity against these pathogens.

Methods

Cell culture and macrophage differentiation

THP-1 monocytic cell line was obtained from ATCC (#TIB-202) and maintained in complete RPMI with 2 mM glutamine and supplemented with 10% heat inactivated fetal bovine serum (C-RPMI). For differentiation into macrophage-like phenotype, cells were resuspended at $8*10^5$ cells/ml and treated with 50 nM phorbol 12-mytistate 13-acetate (PMA) for 24 hours and rested overnight in C-RPMI with 100 µM Ferric Ammonium Citrate (FeAC) and 50 U/ml of human recombinant IFNγ (R&D Systems, MN USA) unless otherwise stated.

Bacterial strains and infection

The strains used in this study were *M. Bovis* BCG$_{\text{Pasteur}}$ (BCG), and *M. tuberculosis*$_{\text{erdman}}$ (Mtb) provided by Dr. Jeffrey Cox (UC Berkley, CA, USA). RFP-BCG$_{\text{pasteur}}$ was provided by Dr. Andrew Mellor (Augusta University, GA, USA). *Listeria monocytogenes* (*L. mono*) was acquired from ATCC (#15313) and clinical isolate *Salmonella enterica* serovar typhimurium (*S. enterica*) was kindly provided by Dr. Mary Hondalus (UGA, GA, USA). Mycobacteria were grown to an $OD_{600} \approx 0.8$a.u. in 7H9 media supplemented with ADC, 5% glycerol and 0.5% Tween 80, and frozen stock at -20°C in 20% glycerol media (v/v). CFU/ml were determined by serial dilution and plating in 7H10 agar media three weeks after freezing. *L. mono* and *S. enterica* were grown to an $OD_{600} \approx 0.8$a.u. in brain-heart infusion (BHI) or Luria-Bertani (LB) broth respectively and frozen stock at -80°C in 20% glycerol media (v/v). Before infection, BCG or Mtb bacilli were passed through a 21G syringe and opsonized for 2h in RPMI with 10% non-heat inactivated horse serum at 37°C with gentle rocking.

For mycobacteria infection, $3*10^5$ PMA-differentiated THP-1 macrophages were incubated in C-RPMI with opsonized bacilli in 48 well plates, at multiplicity of infection of 5 to 10 bacilli per cell, for 2h at 37°C with 5% CO_2. After internalization, macrophages were washed twice with PBS and left on C-RPMI with 50 µg/ml gentamicin and 50 U/ml IFNγ throughout infection. For intracellular bacterial burden quantification, cells were lyzed at indicated timepoints with 0.1% TritonX-100 for 10 minutes and serial dilutions plated in 7H10 agar media. Colony forming units (CFU) were counted twice after 19 to 23 days at 37°C.

L. mono and *S. enterica* infections macrophages were seeded as described above and incubated with non-opsonized bacteria in C-RPMI for 1h at 37°C with 5% CO_2. After internalization, intracellular bacterial burden was determined as described above for mycobacterial infection, in BHI or LB agar plates after 24 hours incubation at 37°C.

RNA extraction and real-time PCR

Total cellular RNA from 10^6 THP-1 macrophages was extracted with TRIzol (Invitrogen, Thermo Fisher Scient., MA, USA) by following the manufacturer’s protocol and reverse transcribed into cDNA using SuperscriptIII First strand cDNA synthesis Kit (Invitrogen, Thermo Fisher Scient., MA, USA) using poly dT_{20} primers. Quantitative PCR (qPCR) was performed using Bio-Rad IQ SYBR green supermix (Bio-Rad, CA, USA) in a iQ™5

Real-Time PCR Detection System. All values were normalized against GAPDH (ΔCT= CT [HAMP] - CT [GAPDH]). Fold change in expression was calculated as $2^{-\Delta\Delta CT}$, where $\Delta\Delta$CT= ΔCT (test sample) - ΔCT (control). The primer sequences for the genes examined were the following: human Hamp, forward, 5=-GGATGCCCATGTTCCAGAG-3=; reverse, 5=-AGCACATCCCACACTTTGAT-3=; human GAPDH, forward, 5=-GCCCTCAACGACCACTTTGT -3=; reverse, 5=-TGGTGGTCCAGGGGTCTTAC- 3=, human FPN, forward, 5=-CACAACCGCCAGAGAGGATG-3=; reverse, 5=-ACCAGAAACACAGACACCGC-3=; Human FTH, forward, 5=-AGAACTACCACCAGGACTCA-3=; reverse, 5=-TCATCGCGGTCAAAGTAGTAAG-3=.

Hepcidin secretion quantification

Hepcidin levels in media supernatants were determined with human hepcidin DuoSet Elisa Kit (R&D Systems, MN, USA), per manufacture recommendations.

Immunofluorescence microscopy

Anti ferroportin and anti-hepcidin antibodies for ferroportin and hepcidin detection were kindly provided by Dr. Tara Arvedson (Amgen, CA USA), and immunofluorescence staining was done as previously descried (Ross et al. 2012).

Briefly, $2*10^5$ THP-1 macrophages were grown and differentiated in eight- or 16-well chamber microscopy slides and infected as described above, fixed with 4% PFA overnight and permeabilized with 0.1% Tripton X-100. For ferroportin staining, cells were incubated with 2 µg/ml mouse antibody diluted in C-RPMI overnight. For detection cells were incubated with 2 µg/ml goat anti-mouse alexa-fluor-488 (Invitrogen, Thermo Fisher Scient., MA, USA) at 4^oC for two hours. Cells were counterstained with DAPI.

For hepcidin staining, cells were treated with infected, fixed and permeabilized as described above, and stained with 2 μg/ml mouse anti-hepcidin antibody overnight at 4ºC.

Slides were imaged in a Zeiss Axiovert 200M microscope at 40X and 63X and images acquired with Axiocam MRm grey scale camera.

Prussian Blue for iron staining

$4*10^5$ THP-1 macrophages were grown and differentiated in 8-well chamber microscopy slides as described above. After infection cells were fixed with 4% formaldehyde in PBS for 10 minutes at room temperature, washed with PBS and stained twice with 4% hydrochloric acid and 4% potassium ferrocyanide (1:1, v/v) solution of for 25 min (Polysciences Prussian blue stain KIT). After washing with PBS cells were counterstained with filtered 1% Nuclear Fast red solution for 5 to 10 min. After gentle washing with PBS and water, slides were mounted and imaged with Olympus Bx41 microscope and images were acquired with Olympos DP71 color camera at 20 and 40X and 100X lenses and images processed with cellSens v1.14.

Image analysis

Image analysis and mean pixel fluorescence intensity was determined with Zeiss Axiovision Rel 4.8.1 software. Colocalization and Prussian blue staining was quantified with image J 1.51K software. Grey scale images were threshold for background and converted to binary files for automatic shape analysis. Bacilli-protein colocalization was determined as shapes with double positive pixels. Protein-protein colocalization was determined by double positive pixel areas.

Prussian blue staining was quantified in 200x color image thresholds for background and determined as percentage of blue pixel area over total pixel area averaged from at least four different fields from three independent experiments.

Statistics

All data are presented as means ± SD. Statistical significance differences between groups was determined using Student's *t*-test with GraphPad Prism software (CA, USA).

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Authorship

Contribution: R.A. designed experiments, interpreted data, prepared figures and wrote the manuscript; F.Q provided guidance, intellectual input, helped write the manuscript and reviewed the manuscript, P.G. provided guidance, intellectual input, helped write the manuscript and reviewed the manuscript.

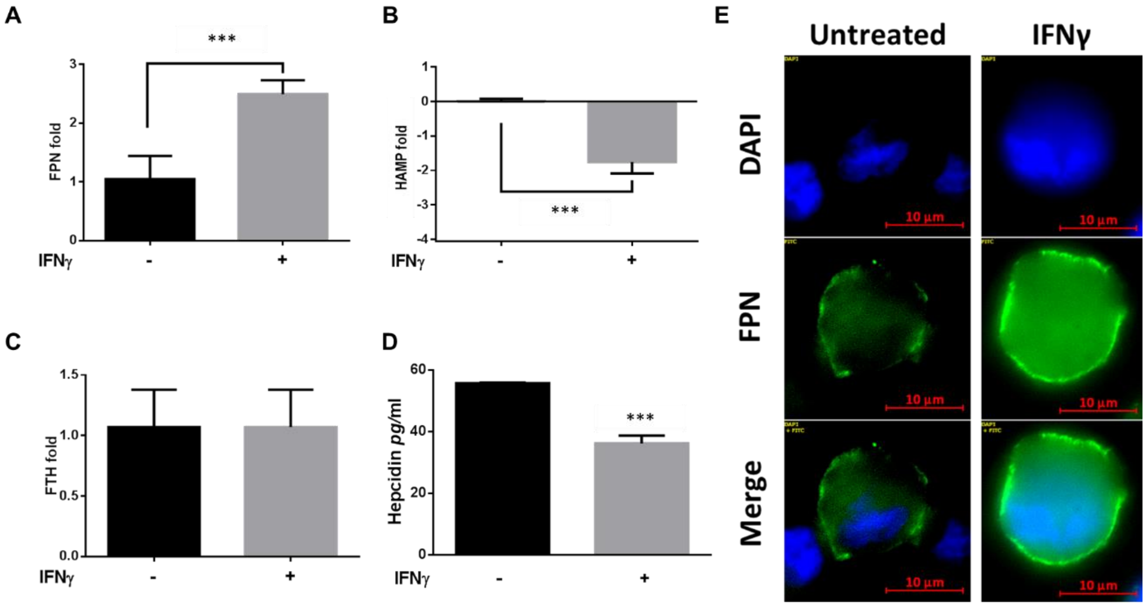

Fig 6.1: **IFNγ regulates iron-related genes to favor iron export.** Transcriptional expression levels of hepcidin (A), ferroportin (B) and ferritin (C) in THP-1 macrophages treated overnight with 200 U/ml IFNγ measured by qRT-PCR and compared to untreated controls. D) Hepcidin secretion in THP-1 macrophages treated as in A. E) Ferroportin expression in THP-1 macrophages treated as in B. **p<0.01, ***p<0.001. All data were from 3 independent experiments.

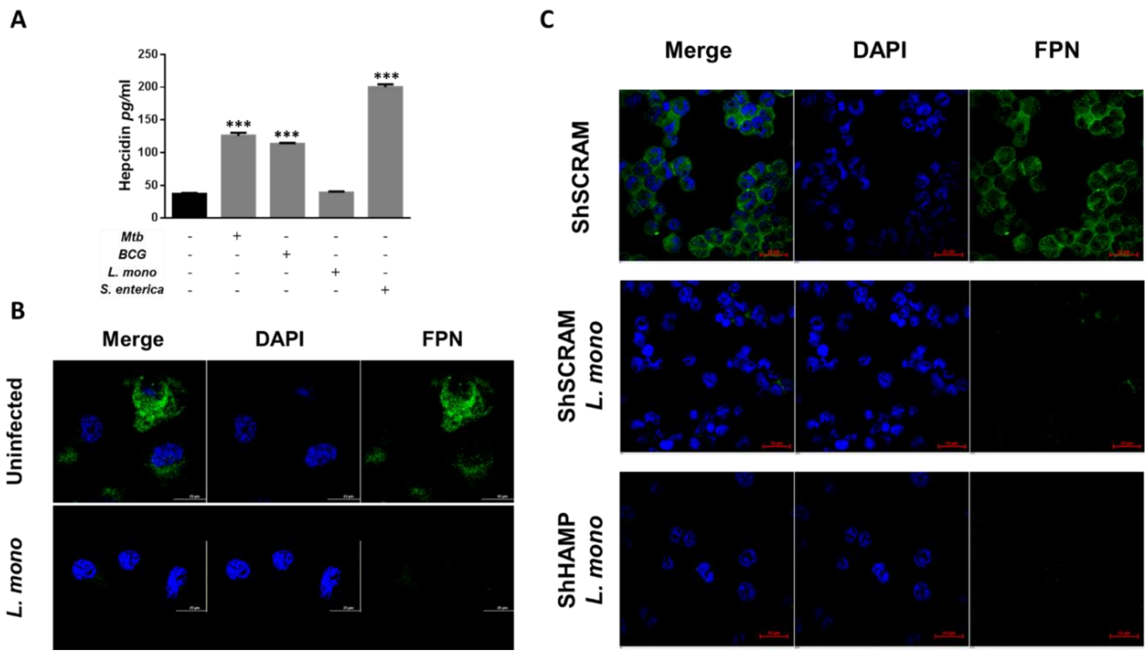

Fig 6.2: **Intracellular pathogens modulate iron-related proteins to favor intracellular iron sequestration in macrophages.** A) Hepcidin secretion from THP-1 macrophages after infection with Mtb (24h), *M. bovis* BCG (24h), *L mono* (8h) or *S. enterica* (16h). B) Ferroportin levels in THP- 1 macrophages eight hours post infection with *L. mono.* C) Ferroportin expression in hepcidin-silenced THP-1 macrophages 8h post infection with *L. mono.* Hepcidin gene silencing in THP-1 cells was achieved by lentiviral based shRNA transduction and Scramble short hairpin RNAs (ShScram) were used as a negative control. ***p<0.001. All data were from 3 independent experiments.

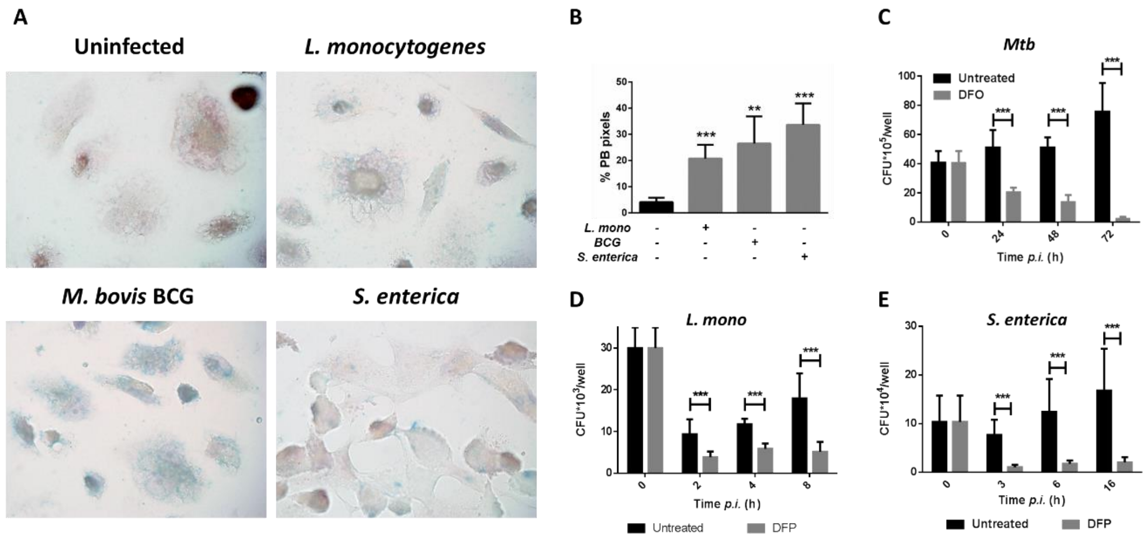

Fig 6.3: **Pathogen associated intracellular iron sequestration facilitates bacterial replication.** A) Intracellular iron Prussian blue staining in macrophages infected with three siderophilic bacteria. B) *BCG* intracellular burden in THP-1 macrophages in presence of iron chelator DFO. C) *L. monocytogenes* intracellular burden in THP-1 macrophages in presence of iron chelator DFP. D) Percentage of Prussian blue pixels in THP-1 macrophages after infection with three siderophilic bacteria. E) *S. enterica* intracellular burden in THP-1 macrophages in presence of iron chelator DFP. ***p*<0.01, ****p*<0.001. All data were from 3 independent experiments.

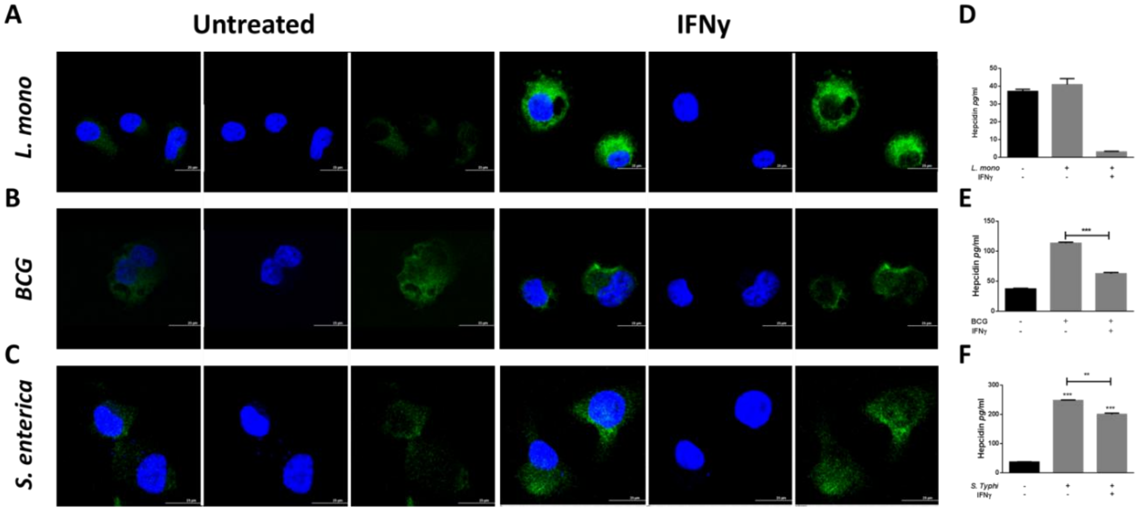

Fig 6.4: **IFNγ prevents pathogen associated iron modulation in macrophages.** A-C) Ferroportin expression in IFNγ-activated (50 U/ml) macrophages after infection 8 hours with *L. mono* (A), 48 hours with BCG (B), or 16 hours with S. *enterica* (C). D-F) Hepcidin secretion in the medium supernatants of IFNγ-activated macrophages after infection 8 hours with *L. mono* (D), 24 hours with BCG (E), or 16 hours with S. *enterica* (F). **p<0.01, ***p<0.001. All data were from 3 independent experiments.

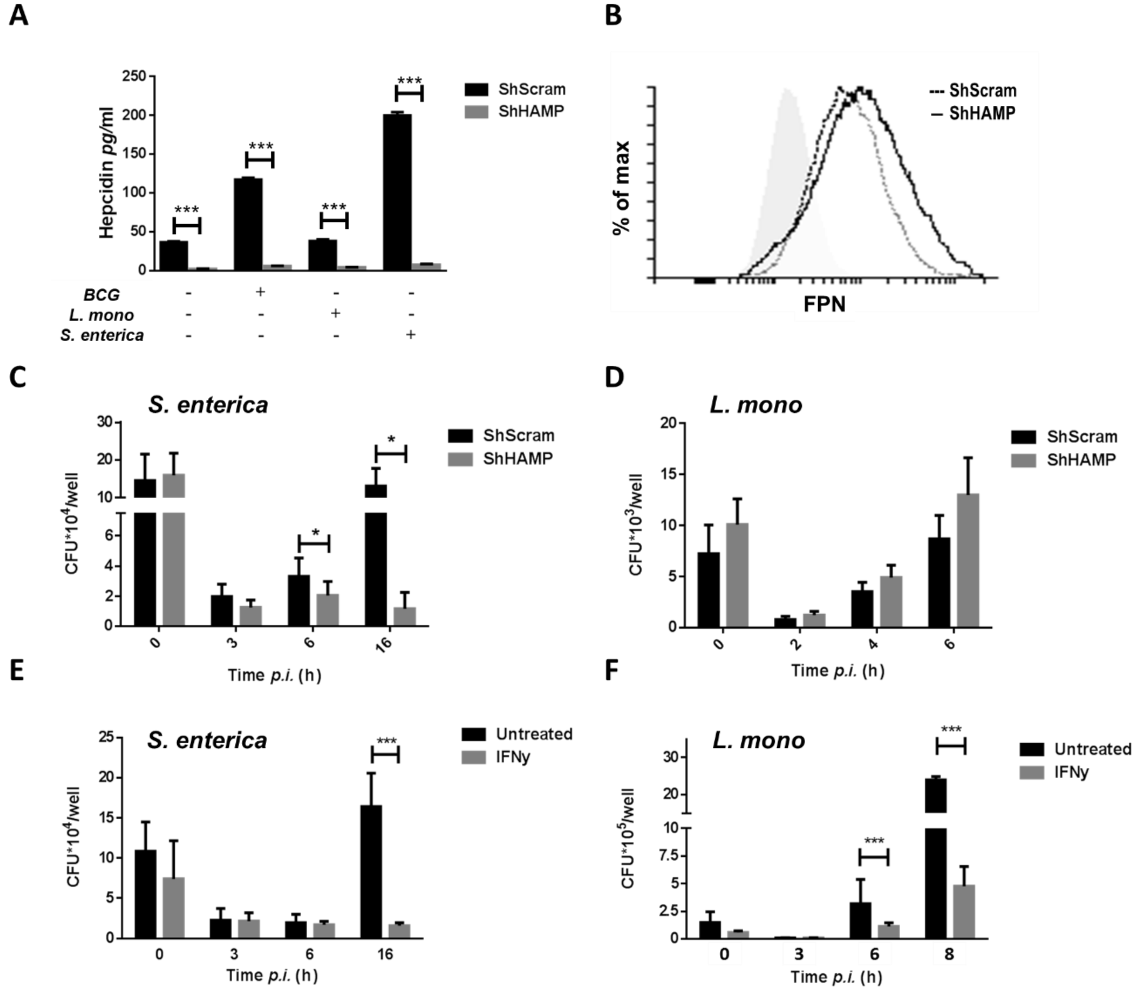

Fig 6.5: **Hepcidin inhibition limits intracellular *Salmonella* replication in macrophages.** A) Hepcidin secretion by hepcidin-silenced (ShHAMP) THP-1 macrophages and respective scramble controls (ShScram) after infection with *BCG*, *L. monocytogenes* and *S. enterica*. B) Surface ferroportin expression HAMP silenced (ShHAMP) THP-1 macrophages and respective scramble controls (ShScram) measured by flow cytometry. C and D) *L. monocytogenes* (C) and *S. enterica* (D) intracellular burden in ShHAMP THP-1 macrophages and respective ShScram controls. E and F) *L. monocytogenes* (E) and *S. enterica* (F) intracellular burden in IFNγ-activated macrophages (50 U/ml and respective untreated controls. *p<0.05, ***p<0.001. All data were from 3 independent experiments.

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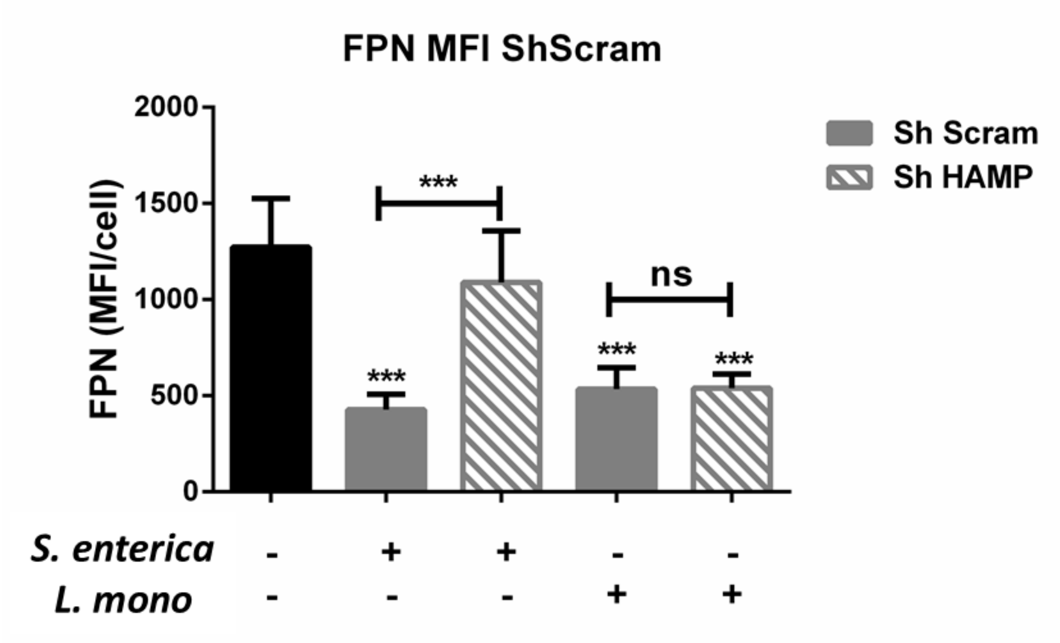

Fig S6.1: **Listeria downregulates ferroportin by a hepcidin-independent mechanism.** Ferroportin expression in hepcidin silenced THP-1 macrophages 8 hours post-infection with *L. mono* and 16 hours post-infection with *S. enterica*. Ferroportin levels were quantified by mean fluorescence intensity of 40 cells from three different fields of three independent experiments. $^{***}p<0.001$

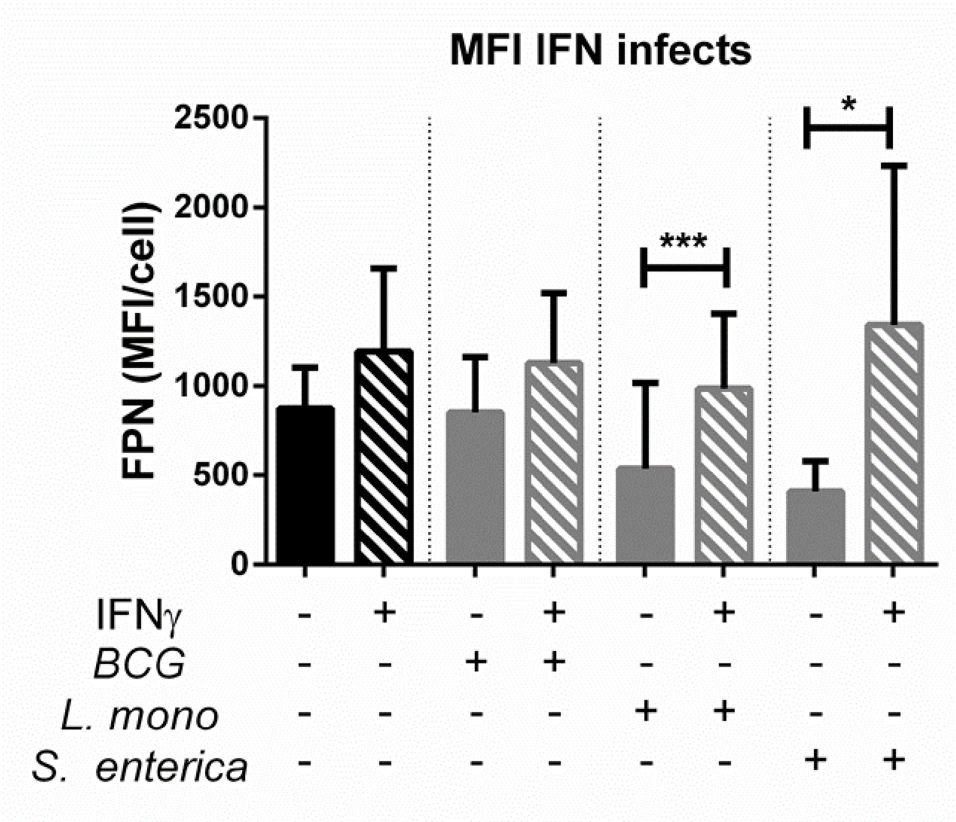

Fig S6.2: **INFγ prevents pathogen-associated ferroportin downregulation.** Ferroportin in IFNγ-treated THP-1 macrophages 8 hours post-infection with *L. mono,* 16 hours post-infection with *S. enterica* and 24 hours post-infection with *BCG*. Ferroportin levels were quantified by mean fluorescence intensity of 40 cells from three different fields of three independent experiments. *p<0.05, ***p<0.001

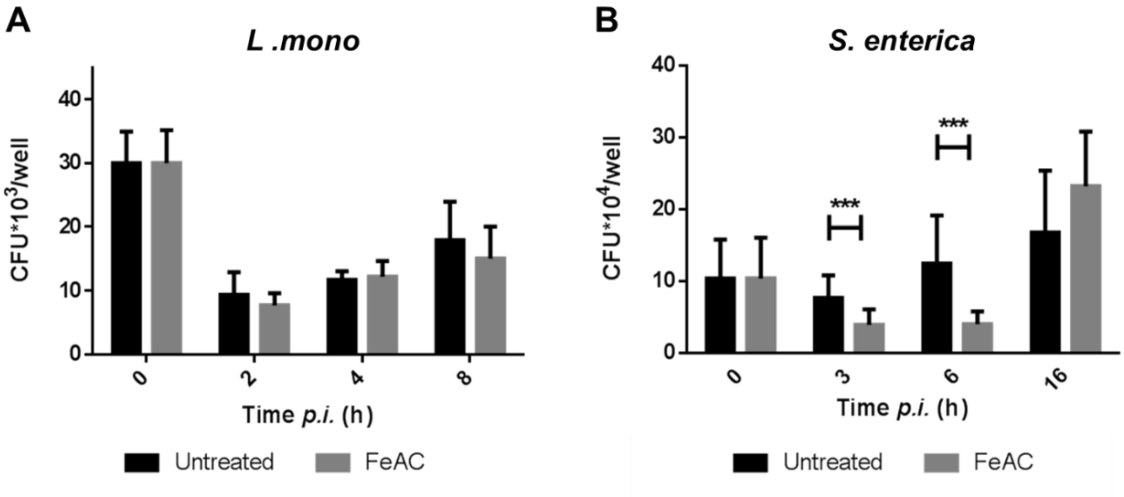

Fig S6.3: **Iron impacts siderophilic bacteria intracellular replication in macrophages.** A) THP-1 macrophages differentiated as described in material and methods, rested and infected in iron supplemented media. *L. mono* (A) and *S. enterica* (B) intracellular bacterial burden was determined by a gentamicin protection assay and CFU enumerated at different time points. ***$p<0.001$. Data from three independent experiments

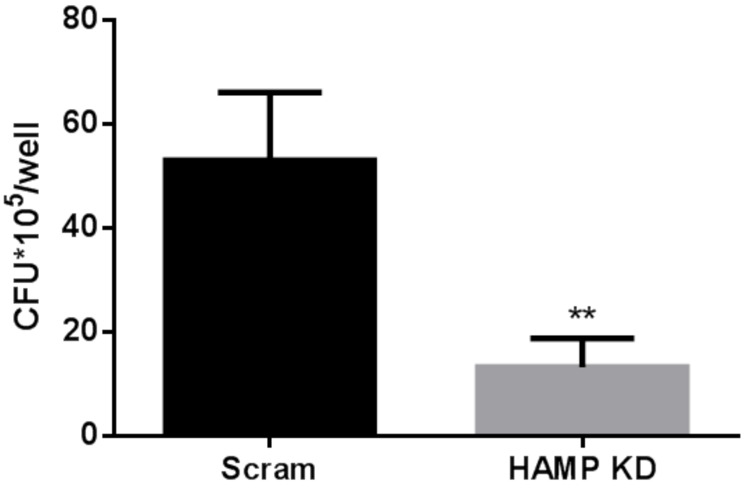

Fig S6.4: **Hepcidin promotes *Mycobacteria* intracellular replication.** BCG intracellular burden in hepcidin silenced THP-1 macrophages 24 hours post-infection. $**p<0.01$. Data from three independent experiments.

CHAPTER 7

HEPARIN DECREASES INTRACELLULAR IRON LEVELS IN MACROPHAGES TO LIMIT *MYCOBACTERIUM TUBERCULOSIS* REPLICATION

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[1] Abreu R, Quinn F., Giri P. Accepted in Scientific Reports, April 13th 2018, Republished here with permission of Scientific Reports

Abstract

Iron is a crucial micronutrient for both mammals and their associated pathogens, and extensive literature has shown that *Mycobacterium tuberculosis* (Mtb) bacilli inhibited from acquiring iron from the host are severely attenuated. In contrast, increased dietary iron concentrations or patients with hemochromatosis have long been associated with a more severe tuberculosis (TB) disease outcome.

We have observed that upon macrophage infection, Mtb bacilli strongly promote intracellular iron sequestration, both through increased expression of hepcidin, a key mammalian iron regulatory protein, and downregulation of the iron exporter protein, ferroportin.

Heparin is a highly sulfated glycosaminoglycan released by mast cells and basophils at sites of tissue injury. During Mtb infection, heparin alters intracellular trafficking in alveolar epithelial cells and decreases extrapulmonary dissemination but recently, heparin also has been reported to inhibit hepcidin expression in hepatocytes, decreasing intracellular iron availability.

In this report, we demonstrate that heparin significantly reduces hepcidin expression in macrophages infected with Mtb bacilli. Heparin-treated macrophages have higher ferroportin expression compared to untreated macrophages, promoting iron export and decreasing iron availability to intracellular bacilli. Thus, here we describe a novel immunomodulatory effect and potential therapeutic role for heparin against mycobacterial infection in human macrophages.

Introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), infects nearly 10 million people annually and causes approximately 1.5 million fatalities globally. Despite extensive efforts to control and eradicate TB, we are still failing to meet the milestones of the WHO *End TB strategy*. One-third of the world population is estimated to be latently infected with Mtb with a 10% lifetime risk of reactivation. However, for immunocompromised patients the risk increases to a 10% chance of disease progression every yearReference 1.

Mycobacterium tuberculosis is one of the most prevalent human pathogens that has evolved to persist in alveolar macrophages ultimately causing extensive lung inflammation and pathology[2,3]. Macrophages serve as the major intracellular niche for Mtb. Upon successful infection, Mtb bacilli evade the macrophage innate antimicrobial functions, inhibit the phagolysosome fusion process and gain access to crucial intracellular nutrients[4]. Inhibition of the inflammasome and impaired IL-1β secretion is associated with increased intracellular bacterial proliferation[5]. Alternatively, chelation of intracellular nutrients such as iron strongly inhibits Mtb replication in macrophages[6,7]. Iron dysregulation has been strongly associated with worsened disease outcomes in Mtb infected patients[8], while effective iron export in macrophages decreased intracellular mycobacterial replication[9].

Heparin is a highly sulfated glycosaminoglycan released by mast cells and basophils at sites of tissue injury. Despite its well-described anticoagulant activity, heparin's physiological role in innate immunity during infection is not fully understood[10]. The mycobacterial adhesin heparin-binding hemagglutinin (HBHA) is an important virulence factor for adhesion, internalization and dissemination from the lung during Mtb infection[11,12]. Heparin and other glycosaminoglycans can decrease the *Mtb* bacterial burden in epithelial cells, but its impact in intracellular replication in macrophages has not yet been investigated. Heparin has multiple modulatory effects on the host cells[13]. For example, heparin has been implicated in multiple biological processes and is capable of interacting with hundreds of human proteins[14,15]. As an immunomodulatory molecule, heparin has been shown to inhibit complement activation, modify cytokine secretion in human mononuclear cells and inhibit leukocyte recruitment[16–19]. Heparin also has been reported to have some antiviral activity through direct interaction with viral proteins[20,21]. Most studies with heparin have been performed on hepatocytes, where the glycosaminoglycan has been shown to inhibit hepcidin expression, thereby decreasing intracellular iron levels in this iron regulatory cell type[22–25]. Interestingly, we have now observed that upon macrophage infection, Mtb bacilli strongly promote intracellular iron sequestration both through induction of hepcidin and direct down-regulation of the iron exporter ferroportin (unpublished data).

In this study, we report that heparin significantly inhibits hepcidin expression in human macrophages after mycobacterial infection. Heparin-treated macrophages express higher ferroportin surface levels compared to untreated controls, promoting iron export and decreasing iron availability to intracellular bacilli. Similar to iron-chelation treatment, heparin significantly reduces Mtb intracellular replication in macrophages. Bacterial internalization and intracellular viability rates were similar between the heparin-treated and control infections, thus the observed lower replication rate is likely the result of the inability of the intracellular bacilli to sequester iron from their niche.

This study suggests a new immunomodulatory function of heparin in macrophages, and a possible protective mechanism for sulfated glycosaminoglycan during Mtb infection. The outcome of this study also provides impetus for screening and assess of modified heparins as novel immunomodulatory anti-mycobacterial therapeutic molecules.

Results

Heparin decreases mycobacterial intracellular replication in human macrophages

Heparin-binding hemaglutinin protein (HBHA) may be an important adhesin for effective attachment of Mtb bacilli to alveolar epithelial cells[12]. However, other than binding to HBHA and interfering with attachment to these epithelial cells, the roles for heparin and other sulfated glycosaminoglycans in *Mtb* intracellular replication and survival have not been tested. *Mycobacterium bovis* BCG is an avirulent vaccine strain frequently used as a BSL2 model to study Mtb replication in macrophages. To assess the impact of heparin on BCG internalization and intracellular replication, THP-1 macrophages were treated overnight with 50µg/ml (≈10U/ml) heparin and infected with opsonized BCG at a MOI of 10. After two hours, bacterial uptake was similar between the heparin-treated and untreated macrophages (p=0.792); however, by 24 hours post infection, heparin-treated macrophages showed a significant 50.6% (±6.97) reduction in intracellular bacterial numbers when compared to untreated controls (p=0.006, fig 1A and B). Because BCG is an avirulent strain of *M. bovis*, intracellular replication is limited compared to fully virulent strains in human macrophages. Thus, after 48 hours, intracellular replication stops for control BCG infections, unrelated to the heparin treatment (fig 1B).

Considering the reduction in early intracellular BCG replication, we assessed and compared the impact of heparin treatment on Mtb-infected macrophages. As observed with BCG, heparin-treated and control macrophages showed no differences in Mtb uptake two hours after internalization (*p*=0.556, fig 1C). However, by 48 hours post infection, heparin-treated macrophages showed significantly decreased intracellular bacterial burdens compared to untreated controls (*p*=0.045, fig 1C and D). By 72 hours post infection, heparin treatment continued to decrease the intracellular bacterial burden compared with untreated controls, reaching a 64% (±5.807) decrease (fig 1D). Furthermore, heparin treatment significantly improved macrophage cell viability by 72 hours after Mtb infection (mean difference was 28±4.082%, fig1E). Altogether, these data point to a host-protective role of heparin during Mtb infection by limiting intracellular bacterial burden in macrophages.

Heparin treatment does not affect bacterial viability *in vitro.*

Heparin antimicrobial activity against Gram-positive bacteria has been long reported [26], but its impact on the growth of *Mycobacterium* species has not been evaluated. When 7H9 medium was supplemented with 50µg/ml of heparin, replication rates were not affected as measured by changes in absorbance patterns (OD_{600}) (fig 2A). Since the effects could be exacerbated in a hostile environment such as within macrophages, increasing heparin concentrations (up to 250µg/ml) in 7H9 medium also was assessed, but no changes in BCG growth were observed compared to untreated broth (fig 2B).

Heparin interacts with a myriad of serum proteins including complement factors [15]. During infection, heparin was added to complete RPMI medium with heat inactivated FBS. To evaluate if heparin is promoting Mtb killing through interaction with serum-proteins, 2.5X10^6 bacilli were incubated in C-RPMI for 72 hours in the absence of macrophages. Aliquots were spread onto 7H10 agar plates, and viable counts assessed. Again, heparin showed no direct bactericidal activity in C-RPMI medium (p=0.216, fig S1B).

Altogether, these data demonstrate that heparin decreases Mtb replication through an indirect mechanism dependent on intracellular macrophage activity.

Heparin treatment does not affect bacterial internalization.

Heparin treatment has no impact on overall viable bacterial uptake compared to untreated control cells (fig 1A and C). However, the impact of heparin treatment on the percentage of host cells infected also was assessed. In these studies, heparin-treated macrophages were infected with red-fluorescent protein (RFP) labeled BCG and the percentage of infected cells was quantified by fluorescence microscopy two hours after internalization (fig 2D). In agreement with the total uptake results, heparin-treatment had no impact on the percentage of infected macrophages (p=0.3*)* (fig 2C).

These data indicate that heparin's impact on the intracellular mycobacterial burden is independent of its previously reported role in bacterial attachment and dissemination[11]**.**

Heparin induces IL-1β secretion during Mtb infection

IL-1β has been well correlated with a protective response to Mtb infection, thus the secretion of this cytokine was assessed in heparin-treated macrophages[5]. Heparin treatment alone does not induce IL-1β secretion in macrophages, although, 24 hours after Mtb or BCG infection, heparin-treated macrophages secrete significantly more IL-1β compared to untreated controls (mean difference was 38.38±2.443 *pg*/ml, fig 3A). Infection with Mtb bacilli is known to induce IL-1β both through inflammasome-

(NLRP3) dependent and independent pathways[27]. Thus, the contribution of heparin towards the induction of IL-1β was assessed after stimulation with NLRP3 specific ligands such as nigericin and ATP. Despite a marginally significant increase in IL-1β secretion by heparin-treated macrophages after LPS and nigericin treatment (mean difference was 12.79±3.054 *pg*/ml, p=0.014, fig S2A), Mtb infection generated a more robust response (mean difference was 529.90±11.16 *pg*/ml, fig 2A). In contrast, ATP-induced IL-1β is not affected by heparin treatment (p=0.181, fig S2A), and heparin could not act as a sole first or second signal for inflammasome activation (fig S2B).

To confirm that heparin's protective role during Mtb infection was dependent on IL-1β secretion, the impact of heparin treatment using caspase recruitment domain- (ASC)-deficient THP-1 (THP-1 ASC^{def}) macrophages was assessed during Mtb infection. THP-1 ASC^{def} macrophages have impaired inflammasome activation and decreased IL-1β secretion after Mtb infection (fig 2B). In accordance with the previously reported protective effect of IL-1β, Δ_{ASC}THP-1 macrophages show increased intracellular bacterial replication compared to parent THP-1 cells (p=0.013, fig 1C). Surprisingly, after heparin treatment, THP-1 ASC^{def} macrophages still showed a decreased intracellular bacterial burden at 48 and 72 hours post infection compared to untreated cells. In fact, when normalized to the respective untreated controls (fig 2E), THP-1 ASC^{def} heparin-treated macrophages supported increased bacterial replication in relation to parent heparin-treated cells at 24 hours (p=0.015), but by 48 and 72 hours post infection, intracellular bacterial replication was inhibited to similar levels in both cell lines (p= 0.93, fig 2E). These data suggest that heparin-induced IL-1β secretion is not responsible for the major differences in bacterial burden observed at later time points (48-72 hours) ion our infection model.

Heparin inhibits hepcidin expression in THP-1 macrophages

Heparin is a known inhibitor of hepcidin expression in hepatocytes[22] and hepcidin has been associated with increased replication of intracellular pathogens [28,29]. To test if heparin can inhibit hepcidin expression in macrophages, THP-1 macrophages were treated with 50µg/ml heparin overnight and hepcidin mRNA expression was measured by qRT-PCR.

TLR4 activation induces hepcidin expression in macrophages [30,31]. To see if heparin could inhibit TLR-mediated hepcidin induction, heparin-treated macrophages were stimulated with LPS (500ng/ml) for 24 hours and hepcidin gene transcription was assessed by qRT-PCR. These data were in accordance with a previous report that showed LPS treatment induces hepcidin expression in macrophages (13.61±3.76 fold), however this response was not significantly affected by the addition of heparin (p=0.3406, fig S3A).

We previously observed that iron supplementation greatly enhances TLR4-mediated hepcidin expression (unpublished data), thus the ability of heparin to inhibit hepcidin expression was assessed under these conditions (fig S3B). When grown in FeAC supplemented medium, LPS-stimulated macrophages showed a 39.78-fold (±3.53) induction in hepcidin mRNA levels compared to controls (p=<0.001); however, heparin-treated macrophages expressed significantly less hepcidin expression when stimulated under the same conditions (mean difference was 35.68±3.56). In fact, LPS-mediated hepcidin induction is lower in heparin-treated macrophages grown in iron-supplemented versus iron-free media (fig S3B).

BCG and Mtb activate TLR4 signaling, and have been shown to induce hepcidin in iron-supplemented medium (unpublished data). Thus, the inhibition of BCG-induced hepcidin expression in THP-1 macrophages also was assessed. After BCG infection, heparin-treated macrophages showed decreased hepcidin mRNA levels compared to untreated controls (mean difference was 28.72±2.32 fold, figs 4A, B and S5). Consistently, hepcidin secretion is decreased four-fold in the culture supernatants from heparin-treated macrophages after BCG and Mtb infection (mean difference was 81.36± 1.235 pg/ml, fig 4D).

Overall, these results show that heparin inhibits Mtb-induced hepcidin expression in macrophages.

Heparin inhibits hepcidin-mediated ferroportin internalization and degradation

Secreted hepcidin binds to the iron exporter protein ferroportin, leading to its internalization and degradation [32]. Heparin can prevent LPS-mediated hepcidin expression and consequent ferroportin internalization in macrophages (fig S4). Like LPS treatment, BCG infection leads to decreased surface ferroportin levels 48 hours after infection, which overlaps with maximum differences in hepcidin expression (fig 5A). Quantification of mean pixel fluorescence intensity shows that infected cells express 44% less ferroportin and 16 times more hepcidin than uninfected controls (fig 5B). Interestingly, 48 hours after infection, 75.8±0.02 % of intracellular BCG bacilli in macrophages would co-localize with ferroportin, although in heparin-treated macrophages only 43±0.5 % of BCG bacilli overlap with ferroportin staining (fig 5C and

S6). This shows that heparin inhibits hepcidin-mediated ferroportin internalization and degradation.

Heparin decreases intracellular iron levels

Increased intracellular iron is normally associated with increased ferritin expression. Similarly, increased hepcidin secretion and decreased ferroportin expression are associated with increased intracellular iron sequestration. Macrophages infected with BCG express higher ferritin levels than uninfected controls, also suggesting increased intracellular iron sequestration (fig 6A and S7A and B). Heparin treatment can slightly decrease ferritin expression after BCG infection but not to levels that resemble uninfected cells (figs 6A and S7A and B).

Mycobacterium tuberculosis and BCG can sequester iron from cytoplasmic iron storage compartments of infected macrophages. In this study, intracellular ferritin distribution was assessed after BCG infection. Consistent with western blot data, BCG-infected macrophages have higher levels of ferritin compared to uninfected controls resulting from increased intracellular iron sequestration (fig 6A). Surprisingly, in uninfected macrophages ferritin localizes to the nucleus, with very little distribution in the cytoplasm. Upon BCG infection, ferritin is found in the cytoplasm (fig 6C). This can be confirmed microscopically by strong association between infecting bacilli and labeled ferritin (87.6±1.7% ferritin-positive intracellular bacilli, fig 6B). Heparin has no impact on intracellular ferritin levels in BCG infected macrophages, but it seems to alter its intracellular distribution with increased nuclear localization (fig 6C) and decreased association with the BCG bacilli (31.1±2.1%, fig 6B).

Decreased ferritin without increased iron export leads to increased free iron levels which have a complex role in bacterial survival and replication. Free cytoplasmic iron is easily accessible by the intracellular bacteria, but if the iron is transported to the phagolysosome, it contributes to reactive oxygen species production through the Fenton reaction which strongly promotes bacterial killing. To evaluate the impact of heparin treatment on the intracellular labile iron pool (LIP), the percentage of calcein fluorescence quenching in heparin-treated macrophages was assessed after LPS stimulation. LPS treatment mimics some aspects of BCG or Mtb infection with strong hepcidin and ferritin induction, ferroportin down regulation and increased intracellular iron levels (fig. S7D). Iron supplementation promotes a moderate but significant increase in the intracellular LIP, however, this is not altered by LPS stimulation until six hours after treatment. After 24 or 48 hours post treatment, LPS-treated cells have much higher intracellular LIP levels, as observed by increased calcein-quenching (67.1% decrease in fluorescence). Nonetheless, LPS-induced LIP is not changed in heparin-treated macrophages suggesting that heparin does not alter intracellular LIP levels. Pam3CSK4, the TLR2/TLR1 activator, induces intracellular iron arrest by direct transcriptional down regulation of ferroportin and through a hepcidin-independent mechanism (unpublished data). Pam3CSK4-treated macrophages show similar intracellular LIP with LPS-treated cells, suggesting regulation of LIP in macrophages is independent of hepcidin expression. Ferritin expression is strongly associated with iron storage levels, but does not represent total iron content of the cell; total intracellular iron levels can be assessed by Prussian blue (PB) staining. Consistent with our previous observations, uninfected macrophages have low iron content, as seen by low staining (fig. 6D). However, upon BCG-infection, intracellular iron content is increased (12.7±5.1%). In contrast, heparin-treated infected macrophages show significantly decreased intracellular iron levels compared to uninfected controls (*p*=0.01) and untreated infected cells (*p*=0.007, fig. 6D). Overall, these results demonstrate that heparin decreases iron availability to intracellular mycobacteria in macrophages.

Heparin can only inhibit intracellular BCG and Mtb replication under high intracellular iron conditions

Mycobacterium bovis BCG replication is generally well contained in human macrophages, although virulence can be promoted by different mechanisms [33]. Iron supplementation promotes enhanced BCG replication in macrophages which can be inhibited by heparin treatment (fig 7B). Furthermore, it has been shown previously that BCG can only induce hepcidin expression in macrophages when grown in iron-supplemented medium (fig 7A), thus, the impact of heparin treatment was assessed in non-iron supplemented medium. When compared to untreated controls, heparin treatment had no impact on intracellular bacterial replication in plain RPMI (no iron added) (fig 7B), further connecting heparin with hepcidin down regulation and decreased iron availability.

Deferiprone is an iron chelator clinically approved for the treatment of iron overload disorders and thalassemia [34–36]. During Mtb infection, treatment with deferiprone significantly decreases intracellular bacterial replication in macrophages (fig 7C). In agreement with heparin’s impact in iron availability to intracellular bacilli, heparin/deferiprone -treated macrophages have similar bacterial burdens to deferiprone -alone treated controls (fig 7C).

Hepcidin supplementation increases intracellular bacilli replication in heparin-treated macrophages

Hepcidin$_{25}$ synthetic peptide is commercially available and has been shown to maintain the iron modulatory function of the native protein [37]. To prove that heparin-mediated hepcidin inhibition is responsible for decreased intracellular bacterial burden at later time points of infection, heparin-treated macrophages were supplemented with hepcidin$_{25}$ synthetic peptide. As predicted, hepcidin supplementation rescued intracellular bacterial replication in heparin-treated macrophages; however, no significant increase in untreated macrophages was detected (fig 7D).

Discussion

Notwithstanding the efforts to eradicate it, tuberculosis has again become the leading cause of death due to an infectious disease [38,39]. The increase in infections with multidrug-resistant and extensively-drug resistant strains makes the use of therapeutics as our only effective intervention strategy a unsustainable plan. Thus, a truly effective control strategy requires that new therapeutics and a more effective vaccines are developed [39]. In this report a protective immunomodulatory role for heparin during Mtb infection in macrophages is described. We demonstrate that heparin modulates macrophage iron status, and decreases iron availability for intracellular bacilli, thus limiting bacterial replication.

Mycobacterium tuberculosis bacilli persist and reside inside alveolar macrophages. To replicate within the phagosome of these cells, Mtb bacilli must recruit essential nutrients, such as iron to this vesicular compartment [40,41]. Despite the important role of macrophages in iron recycling from erythrophagocytosis, free iron is extremely scarce inside the macrophage being rapidly redistributed extracellularly through the iron exporter ferroportin or sequestered by the iron storage protein ferritin.

Mycobacterium tuberculosis bacilli possess a myriad of mechanisms for iron scavenging inside the host. For example, PAMPs activate TLR2 and TLR4 signaling and promote intracellular iron sequestration in macrophages through induction of hepcidin and down regulation of ferroportin (unpublished data). In addition, increased expression of mycobactin and carboxymycobactin siderophores efficiently recruit and scavenge iron for use by the intracellular mycobacteria within the phagosome [42–44]. Carboxymycobactin is the major iron-chelator for both free and protein-bound iron in the macrophage phagosome and cytoplasm [44–46], while surface mycobactin acts as a membrane chelator and iron-transporter recovering iron from carboxymycobactin and host ferritin. Both molecules are essential for iron acquisition and pathogenesis as shown by the severe attenuation of Mtb knock-out strains with impaired siderophore synthesis [47].

In this study, heparin treatment significantly inhibited Mtb-mediated hepcidin secretion (fig 4) culminating in increased ferroportin expression 48 hours after infection (fig 5). Poli et al. have previously shown that heparin can inhibit hepcidin expression in hepatocytes, but it is shown here that this highly sulfated glycosaminoglycan has a similar impact in TRL-mediated hepcidin expression in myeloid cells.

Mycobacterium tuberculosis infection promotes increased intracellular iron sequestration and ferritin expression (fig 6). Reference 42 showed that *Mycobacterium paratuberculosis* mycobactin J (mbtJ) rapidly disperses from the phagosome in host lipid cellular components, accessing the macrophage intracellular iron pool. Iron loaded mbtJ localizes with lipid droplets that are later associated with the phagosome. Here, heparin had no impact in the intracellular labile iron pool (fig S7), but greatly decreased total intracellular iron levels, potentially protein-bound (fig 6D). In macrophages, ferritin mostly localizes to the nucleus with minimal cytoplasmic distribution (fig 6). Ferritin nuclear translocation has been previously reported in murine macrophages during iron overload [48], but its mechanism and function remain unclear. Macrophages infected with BCG bacilli have decreased ferritin nuclear co-localization, despite the increase in ferritin expression (fig 6A), while heparin-treated macrophages show a nuclear ferritin distribution comparable to uninfected cells. Interestingly, heparin also decreases ferritin - BCG co-localization further decreasing iron availability for the intracellular bacilli (fig 6).

Decreased intracellular iron availability through chelation therapy significantly limits intracellular mycobacterial growth [6,49] and fig 7). In our study, heparin impacts intracellular BCG replication in the presence of iron and can be counteracted by the addition of hepcidin to the medium (fig 7), indicating that heparin-mediated hepcidin inhibition and the decrease in intracellular iron availability are the major action mechanisms limiting intracellular replication. *De facto*, a similar mechanism has been described in IFNγ-activated murine macrophages [50,51]. In that report, IFNγ-induced ferroportin expression contributed to efficient control of *Salmonella enterica* intracellular replication [51].

Alveolar macrophages and potentially type II pneumocytes are the primary cell targets during Mtb infection [52]. Heparin is known to prevent mycobacterial attachment to and internalization of type II pneumocytes [11,12]. However, macrophages actively phagocytose opsonized bacteria through Fcγ receptors and the complement receptor. In this study, heparin treatment of macrophages had no impact on bacterial attachment and internalization, but intracellular replication was reduced at later time points compared to untreated controls. Phagocytosis of opsonized Mtb bacilli by resident macrophages in the lung leads to phagosome acidification and lysosome fusion, increased reactive oxygen and nitrogen species, and recruitment of antibacterial peptides culminating in bacterial clearance [53]. Nonetheless, Mtb bacilli activate an arsenal of virulence factors which block efficient macrophage antibacterial functions [53,54]. An example of these Mtb blocking factors include secretion of phosphotyrosine protein phosphatase (Ptpa) immediately upon Mtb macrophage internalization. This protein inhibits host membrane fusion proteins and host V-ATPases required for phagosome maturation and acidification. An additional factor is Mtb nucleoside diphosphate kinase (NdK) which inhibits phagolysosome fusion and NOX2-mediated ROS production [55,56]. It would be interesting to determine if heparin can counteract bacterial factors and impact early events after phagocytosis such as phagosome maturation, lysosome fusion and promote efficient bacterial killing.

The use of porcine unfractionated heparin (UFH) raises limitations to direct translation from our study into a novel therapeutic strategy. UFH is a mixture of heparins of variable sizes with limited bioavailability and extremely variable anticoagulant pharmacological properties [10]. Still, over the last decade extensive efforts have been made to develop modified heparins with improved and more targeted pharmacological activities. Recently, Poli et al [25] revealed that glycol-split non-anticoagulant heparin fractions can mimic intracellular signaling of UFH leading to hepcidin inhibition in hepatocytes. Future studies will examine if these non-anticoagulant heparins can prevent Mtb-mediated iron sequestration in macrophages and limit intracellular bacterial replication while limiting side effects.

Iron dysregulation has been long associated with increased risk of developing TB [57,58]. Moreover, hepcidin serum levels are strongly correlated with *Mtb*– HIV co-infection [59–61]. Hepcidin expression and decreased iron export have been shown to increase HIV replication rates in macrophages [6], reinforcing the importance of this hormone in co-infection. This study leads the way towards a potential use of hepcidin inhibitors such as heparin, as an efficient therapeutic strategy against TB, and a promising prospect for immunomodulatory therapies in HIV-Mtb co-infected patients.

Methods

Cell culture and macrophage differentiation

The THP-1 monocytic cell line was obtained from ATCC (TIB-202), maintained in complete RPMI with 2mM glutamine and supplemented with 10% fetal bovine serum (C-RPMI). For differentiation into a macrophage-like phenotype, $8X10^5$ cells/ml were treated with 50nM phorbol 12-mytistate 13-acetate for 24 hours and rested overnight in C-RPMI with 100µM ferric ammonium citrate (FeAC). When stated, 50 µg/ml heparin (≈10U/ml) was subsequently added to the medium during overnight resting. THP-1 cells deficient in PYD, the CARD Domain Containing (THP-1 ASC^{def}) cells and the parent strain, all were obtained from Invivogen (CA USA), and maintained and differentiated as described above for the ATCC THP-1 original cell line.

Bacterial strains and infection

The strains used in this study were *Mycobacterium bovis* BCG Pasteur, and Mtb Erdman generously provided by Dr Jeff. Cox (UC Berkley, CA USA). Strain BCG Pasteur expressing RFP was generously provided by Dr. Andrew Mellor (Augusta University, GA, USA). Bacteria were grown to an $OD_{600} \approx 0.8$ in 7H9 medium supplemented with ADC, 5% glycerol and 0.5% Tween 80, and aliquots frozen at -80°C. Frozen aliquots were thawed, serially diluted and plated on 7H10 agar medium containing 10% ADC for three weeks at 37°C. Viability was measured as colony forming units/ml (CFU/ml). Prior to infection, BCG or Mtb bacilli were passed through a 21G tuberculin syringe and opsonized for 2 hours in RPMI with 10% non-heat inactivated horse serum at 37°C with gentle rocking.

For infection, $1.5X10^6$, $3X10^5$ or $8*10^4$ PMA-differentiated THP-1 macrophages were incubated in C-RPMI with opsonized bacteria in 12, 48 or 96 well plates respectively at a multiplicity of infection of five to 10 bacilli per host cell for two hours at 37°C with 5% CO_2. After internalization, macrophages were washed three times with warm PBS. After washing, C-RPMI containing 50µg/ml gentamicin and 50µg/ml heparin was added to the infected cells and maintained throughout the experiment. For intracellular bacterial burden quantification, cells were lysed at indicated time points with 0.1% TritonX-100 for 10 minutes and serial dilutions plated on 7H10 agar medium containing 10% ADC. CFUs were counted twice after incubation for 19 to 23 days at 37°C.

Heparin bacteriostatic and bactericidal assay

BCG bacilli were grown in complete 7H9 medium with increasing heparin concentrations (50 to 250µg/ml), in T25 flasks, at a starting $OD_{600} \approx 0.001$. Growth was measured daily by changes in OD_{600} of 100µl aliquots in 96 well flat bottom plates and assayed using *Powerwave* XS2 (Biotek, VT USA).

Mtb Erdman bacilli were grown similarly to BCG, but changes in absorbance were measure in 13mm diameter spec tubes and assayed using a *Spectronic 20+* spectrophotometer.

To assess heparin bactericidal activity, $2.5X10^6$ bacteria were incubated in C-RPMI with 50µg/ml heparin for 72 hours at 37°C with 5% CO_2, and serial dilutions plated on 7H10 agar medium containing 10% ADC. CFUs were counted twice after incubation for 19 to 23 days at 37°C.

RNA extraction and real-time PCR

Total cellular RNA from $1X10^6$ THP-1 macrophages was extracted using TRIzol (Invitrogen, Thermo Fisher Scientific, MA USA) following the manufacturer's protocol and reverse transcribed into cDNA using SuperscriptIII First strand cDNA synthesis Kit (Invitrogen) with poly dT20 primers. Quantitative PCR (qPCR) was performed using Bio- Rad IQ SYBR green supermix (Bio-Rad, CA USA) in a iQ™5 Real-Time PCR Detection System. All values were normalized against GAPDH (ΔCT= CT [HAMP] - CT [GAPDH]). Fold change in expression was calculated as $2^{-\Delta\Delta CT}$, where $\Delta\Delta$CT= ΔCT (test sample) - ΔCT (control). The primer sequences for the genes examined were the following: human Hamp, forward, 5=-GGATGCCCATGTTCCAGAG-3=; reverse, 5=-AGCACATCCCACACTTTGAT-3=; human GAPDH, forward, 5=-GCCCTCAACGACCACTTTGT -3=; reverse, 5=-TGGTGGTCCAGGGGTCTTAC- 3=.

Hepcidin secretion quantification

Hepcidin levels in culture supernatants were determined using the human hepcidin DuoSet Elisa Kit (R&D Systems, MN USA), per manufacturer's recommendations.

Protein extraction and Western blot analysis

For western blot, 1X10^6 cells were cultured in 6-well plates, washed twice with ice-cold PBS and lysed with ice-cold IP lysis for 30 minutes on ice. Cell lysates were further disrupted manually by vigorous pipetting and vortexing. After centrifugation (10,000Xg) for 15 minutes at 4°C, supernatants were collected and stored at -20°C until analyzed.

Total protein content was determined by using the BCA protein estimation assay kit (Pierce, Thermo Fisher Scientific MA USA). Samples (20µg) were mixed with Laemmli buffer (1x final concentration), heated at 70°C for 10 minutes, and proteins were electrophoretically separated on a 15% sodium dodecyl sulfate (SDS)–polyacrylamide gel. The proteins were transferred to a PVDF membrane (Bio-RAD), which was then pre-incubated with blocking solution (5% nonfat dry milk in Tris-buffered saline containing 0.01% Tween20 [TBST], pH 7.4) for one hour, followed by overnight incubation with 1µg of anti-FTH1 (ferritin) rabbit monoclonal antibody (Cell signaling Tech,, MA USA) and 1µg Anti-GAPDH rabbit monoclonal antibody (cell signaling) at 4°C. After primary incubation, the membrane was washed 3x with TBST and incubated for one hour with secondary anti-rabbit HRP conjugated antibody (Cell signaling Tech).

All incubations and wash steps were performed at room temperature except when otherwise stated. Cross-reactivity was visualized by using enhanced chemiluminescence (SuperSignalWestPico; Pierce).

Immunofluorescence microscopy

Anti-ferroportin and anti-hepcidin antibodies for ferroportin and hepcidin detection were kindly provided by Dr. Tara Arvedson, and immunofluorescence staining was done as previously described.

Briefly, $2X10^5$ THP-1 macrophages were grown and differentiated in eight or 16 well chamber microscopy slides and infected as described above, fixed with 4% PFA overnight and permeabilized with 0.1% Triton X-100. For ferroportin staining, cells were incubated with 2µg/ml mouse antibody diluted in C-RPMI overnight. For detection, cells were incubated with 2µg/ml goat anti-mouse alexa-fluor-488 (Invitrogen, Thermo Fisher Scientific. MA USA) at 4^oC for two hours. Cells were counterstained with DAPI.

For hepcidin staining, cells were treated with infected, fixed and permeabilized as described above, and stained with 2µg/ml mouse anti-hepcidin antibody overnight at 4^oC.

Slides were imaged in a Zeiss Axiovert 200M microscope at 40X and 63X and images acquired with Axiocam MRm grey scale camera.

Prussian blue for iron staining

Four-hundred thousand THP-1 macrophages were grown and differentiated in eight well chamber microscopy slides as described above. After infection, cells were fixed with 4% formaldehyde in PBS for 10 minutes at room temperature, washed with PBS and stained twice with 4% HCl and 4% K4[Fe(CN)6] · 3H2O (1:1 v/v) solution for 25 minutes (Prussian blue stain Kit Polysciences, Warrington, PA USA). After washing with PBS, cells were counterstained with filtered 1% Nuclear Fast red solution for five to 10 minutes. After gentle washing with PBS and double-distilled H_2O, slides were mounted and imaged on an Axiovert 40CFL microscope. Images were acquired on a Axiocam MRC5 color camera with 20X and 40X lenses.

Intracellular labile iron pool (LIP) quantification

Intracellular LIP was measured through a calcein quenching assay as previously described and adapted to flow cytometry analysis. Briefly, $3X10^5$ THP-1 macrophages were seeded in 48-well plates and treated with LPS or Pam3CSK4 (synthetic triacylated lipopeptide that activates the TLR2/TLR1 heterodimer) up to 48 hours in iron supplemented medium. At each timepoint, cells were washed twice with warm PBS, stained with calcein-AM (Invitrogen) for 15 minutes at room temperature, washed again with warm PBS, trypsininzed, resuspended in FACS buffer and analyzed by flow cytometry before and after iron chelation with deferiprone (DFP). Quenched fluorescence was determined as percentage of Mean Fluorescence Intensity before iron chelation (xMFI) to 10 minutes after addition of DFP ($xMFI_{DFP}$) ($\frac{xMFI}{(xMFI_{DFP})} \times 100$). Cells grown in non-iron supplemented medium were used as negative controls.

Image analysis

Image analysis and mean pixel fluorescence intensity were determined with Zeiss Axiovision Rel 4.8.1 software. Colocalization and Prussian blue staining were quantified with image J 1.51K software. Grey scale images were threshold for background and converted to binary files for automatic shape analysis. Bacilli-protein colocalization was determined as shapes with double positive pixels. Protein-protein colocalization was determined by double positive pixel areas.

Prussian blue staining was quantified in 200x color image thresholds for background and determined as percentage of blue pixel area over total pixel area averaged from at least four different fields from three independent experiments.

Statistics

All data are presented as means ± SD. Statistical significance differences between two groups were determined using Student's t test or 2-way ANOVA (Bonferroni) for multiple group comparison with GraphPad Prism software (CA, USA).

Acknowledgments

We thank Dr. Tara Arvedson (Amgen Inc., CA, USA) for providing hepcidin and ferroportin monoclonal antibodies. We also thank Shelly Helms for technical assistance in several parts of this project.

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Conflict-of-interest: The authors have no conflict of interest to declare.

Authorship

Contribution: R.A. designed experiments, interpreted data, prepared figures and wrote the manuscript; A. L. and L.E. performed experiments; F.Q provided guidance, intellectual input, helped write the manuscript and reviewed the manuscript, P.G. provided guidance, intellectual input, helped write the manuscript and reviewed the manuscript.

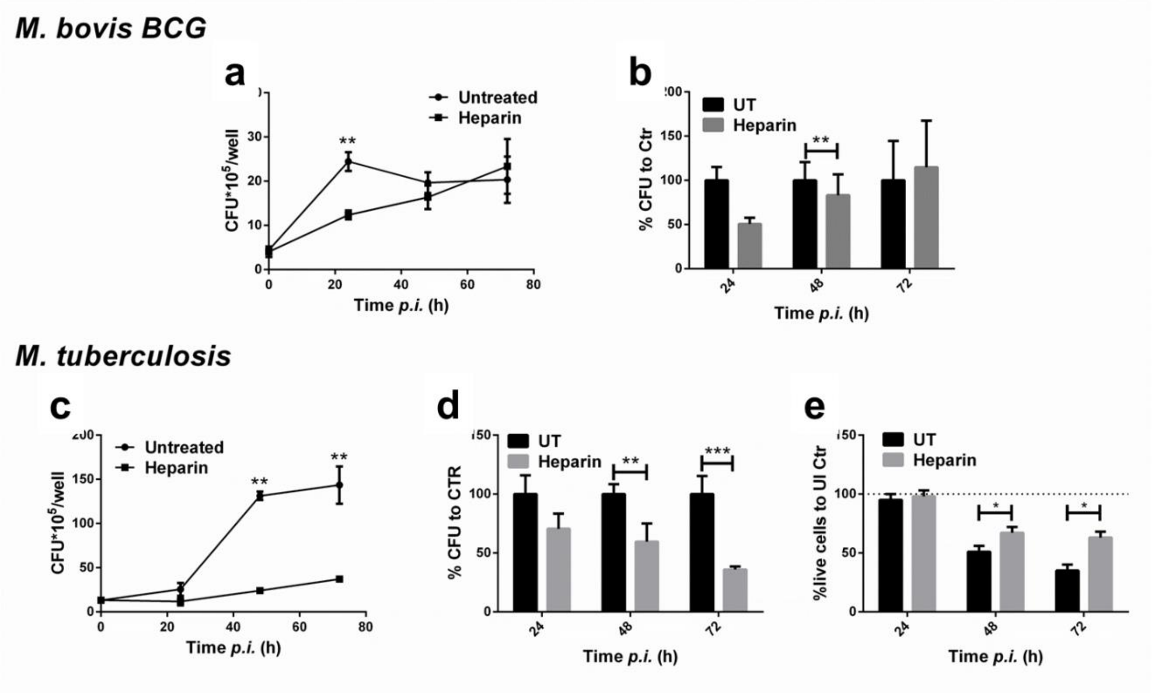

Fig 7.1: **Heparin inhibits Mtb and BCG replication.** a, c) Intracellular CFU in THP-1 macrophages infected with BCG (a) and Mtb (c) at an MOI of 10 after 16 hours of treatment with 50µg heparin. b,d) Percentage of intracellular bacilli in heparin treated macrophages at 24, 48 and 72 hours post infection with BCG (b) and Mtb (d). e) Trypan blue exclusion cell viability in Mtb infected-THP-1 macrophages at 24, 48 and 72 hours post-infection. For a and c macrophages were seeded in 12 well plates Data from three independent experiments. * $p<0.05$, **$p<0.01$, ***$p<0.001$

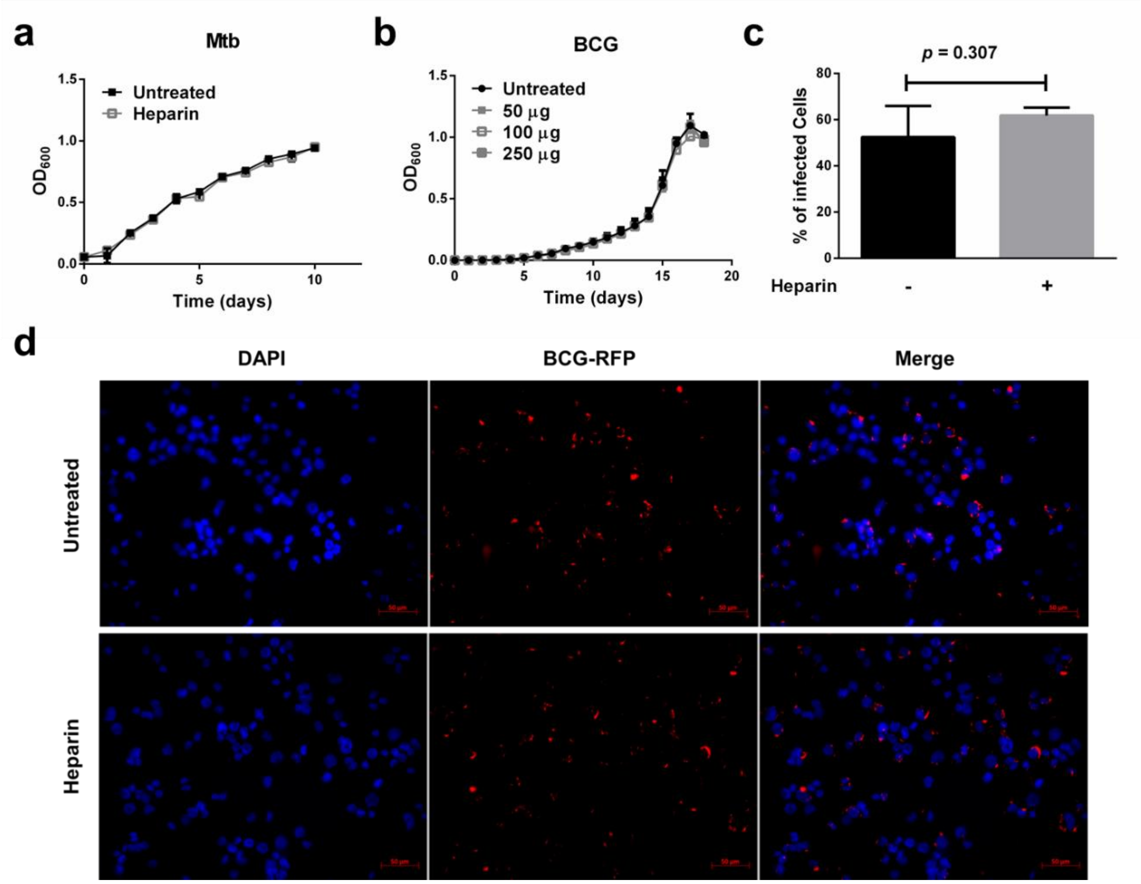

Fig 7.2: **Heparin has no direct impact in bacterial growth or macrophage internalization.** a,b) BCG (a) and Mtb (b) growth in heparin-supplemented 7H9 medium. c) Percentage of infected cells in 9 random fields (10x) from 3 independent experiments as represented in b. d) BCG expressing RFP in THP-1 infected macrophages treated overnight with 50µg/ml heparin two hours after internalization. Data from three independent experiments.

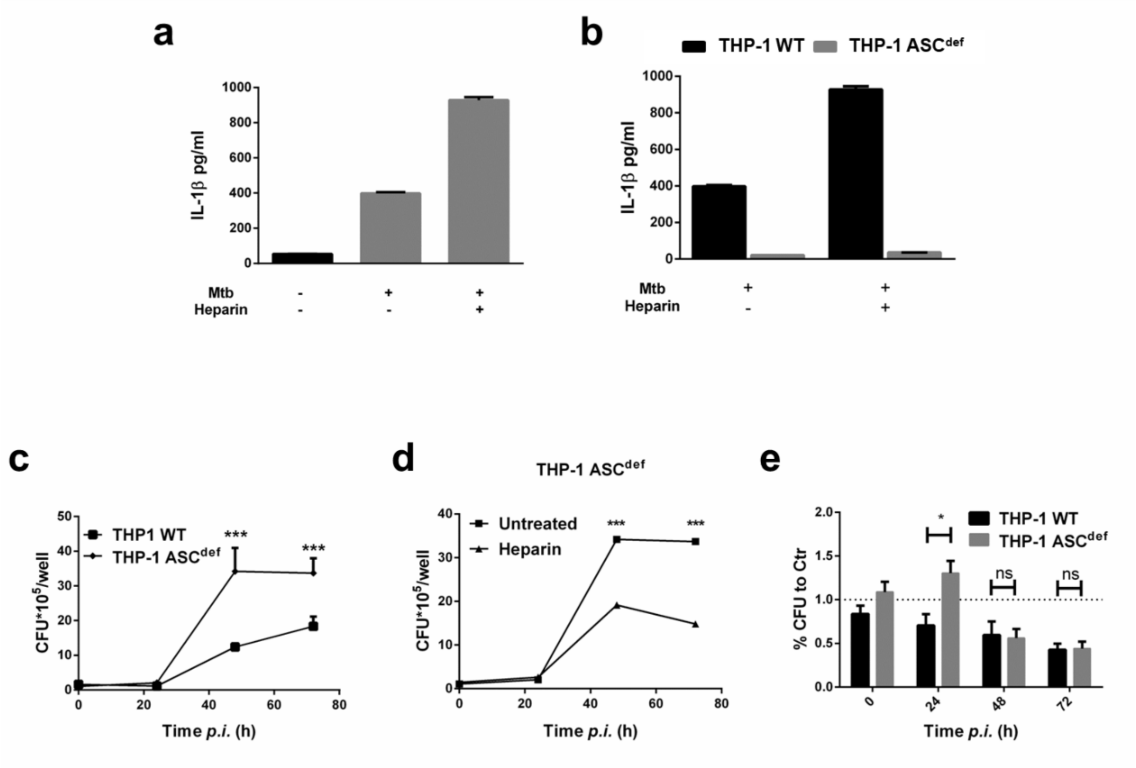

Fig 7.3: **Heparin induces IL-1β secretion during Mtb infection in THP-1 macrophages.** a) IL-1β secretion in culture supernatants of heparin-treated macrophages 24 hours after Mtb infection. b) IL-1β in ASC deficient macrophages 24 hours after Mtb infection. c) Intracellular bacilli in Mtb infected ASC deficient and wild-type THP-1 macrophages. d) Intracellular CFU in heparin-treated ASC deficient THP-1 macrophages. e) Percentage of untreated control intracellular bacterial burden in heparin treated wild-type and ASC deficient THP-1 macrophages. For c and d macrophages were seeded in 48 well plates. Data from three independent experiments. *$P<0.05$, **$p<0.01$, ***$p<0.001$.

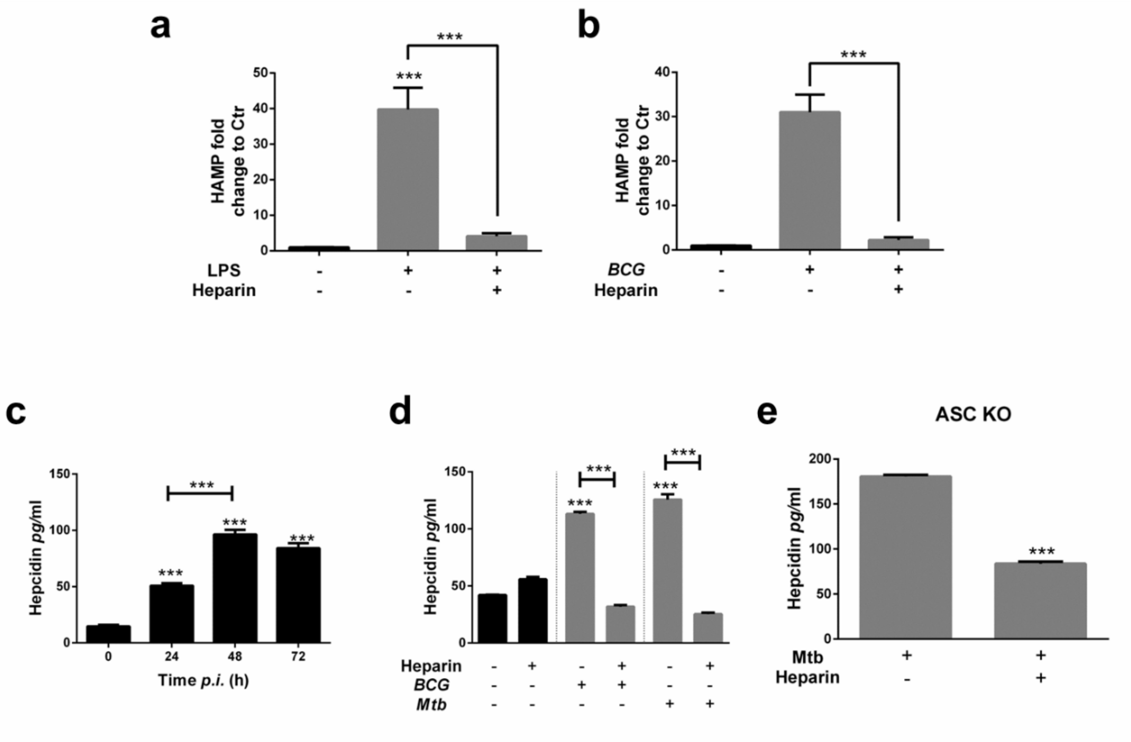

Fig 7.4: **Heparin inhibits hepcidin expression in macrophages.** a-b) Hepcidin expression in heparin-treated macrophages 24 hours after LPS stimulation (a) or BCG infection (b) measured by qRT-PCR. c) Hepcidin secretion in Mtb-infected THP-1 macrophages culture supernatants. d) Hepcidin secretion by heparin-treated macrophages 48 hours after Mtb or BCG infection. e) Hepcidin secretion by heparin treated ASC deficient THP-1 macrophages 48 hours after Mtb infection. Data from three independent experiments. *$P<0.05$, **$p<0.01$, ***$p<0.001$

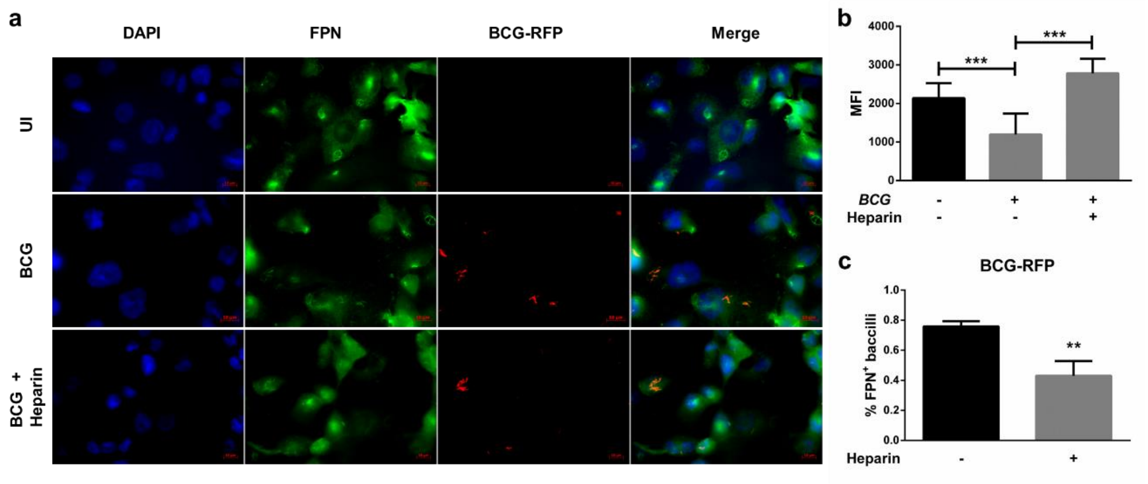

Fig 7.5: **Heparin-treated macrophages have increased ferroportin levels.** a) Ferroportin expression in heparin-treated macrophages 48 hours after BCG infection (63X). b) Ferroportin expression in heparin-treated macrophages 48 hours after BCG infection, measured as Pixel MFI/cell from a minimum of 20 cells in three different fields (40X) from three independent experiments. c) Percentage of BCG bacilli colocalized with ferroportin-FITC staining (overlapping red and green pixels). Data from three independent experiments. ***p<0.01*, ****p<0.001*

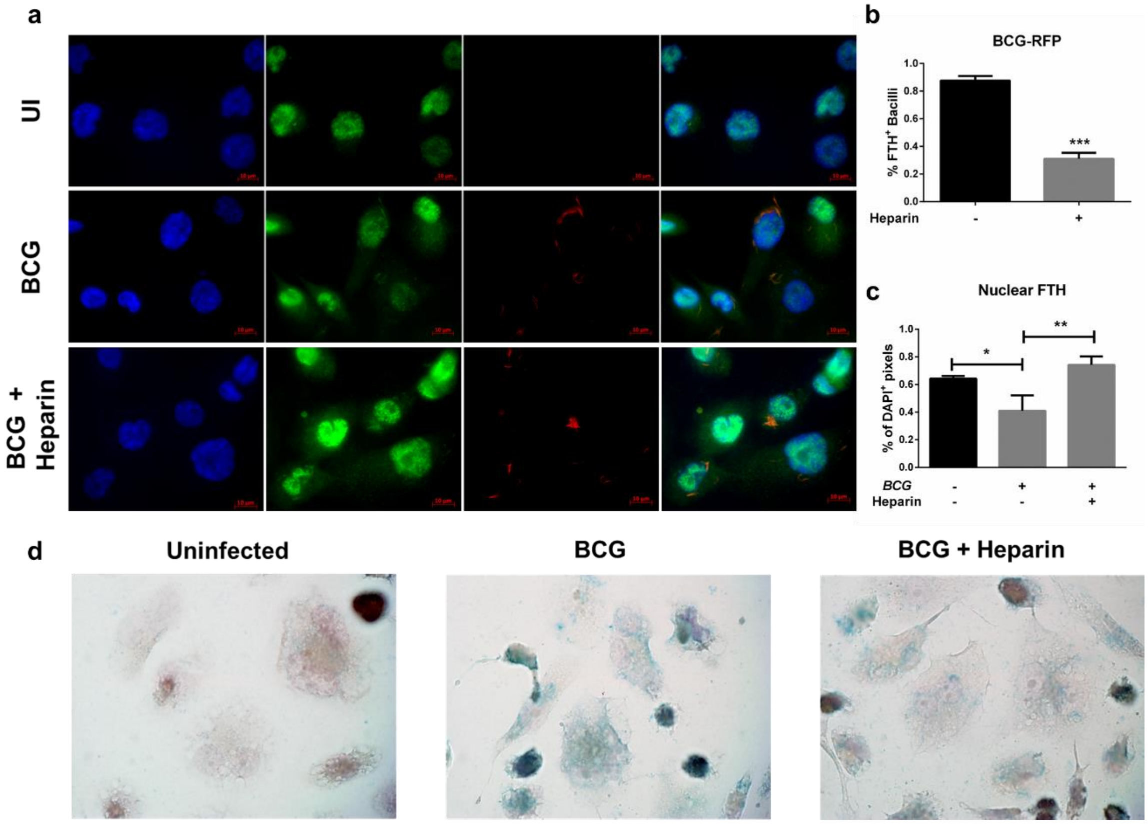

Fig 7.6: **Heparin decreases iron availability to intracellular mycobacterial bacilli.** a) Ferritin expression in heparin-treated macrophages 48 hours after BCG infection. b) Percentage of ferritin -positive BCG bacilli in five fields from three independent experiments as represented in a. c) Nuclear ferritin in heparin-treated macrophages 48 hours after BCG infection measured by percent of double-positive pixels (FITC and DAPI). d) Total intracellular iron staining (Prussian blue) in heparin-treated macrophages 48 hours after BCG infection. Data from three independent experiments. *$*p<0.05$, $**p<0.01$, $***p<0.001$.*

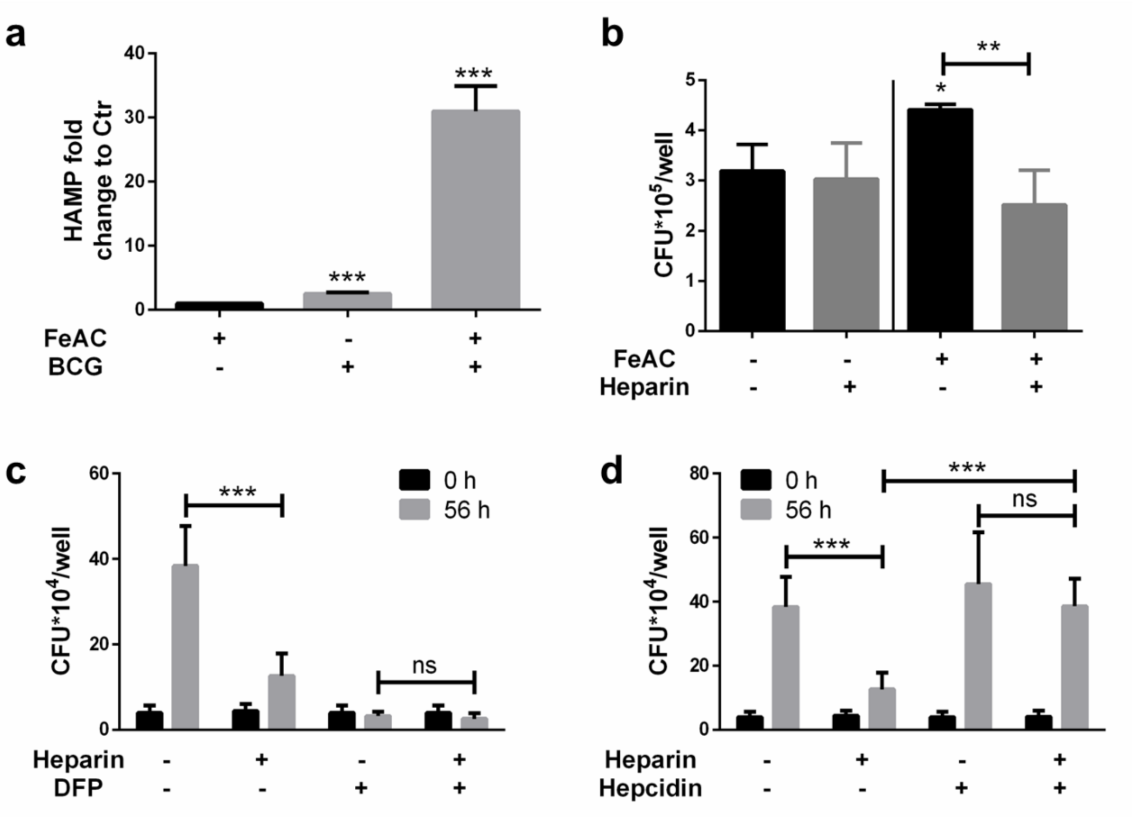

Fig 7.7. **Hepcidin rescues intracellular Mtb replication in heparin-treated macrophages**. a) Hepcidin mRNA expression in BCG-infected macrophages with and without iron supplementation. b) Intracellular bacterial burden in heparin-treated macrophages with and without iron supplementation. c) Intracellular bacterial burden in heparin-treated macrophages in presence of iron chelator deferiprone at 0 and 56 hours post infection. d) Intracellular bacterial burden in heparin-treated macrophages supplemented with hepcidin synthetic peptide. For b macrophages were seeded in 48 well plates. For c and d macrophages were seeded in 96 well plates. Data from three independent experiments. *$p< 0.05$, **$p<0.01$, ***$p<0.001$.

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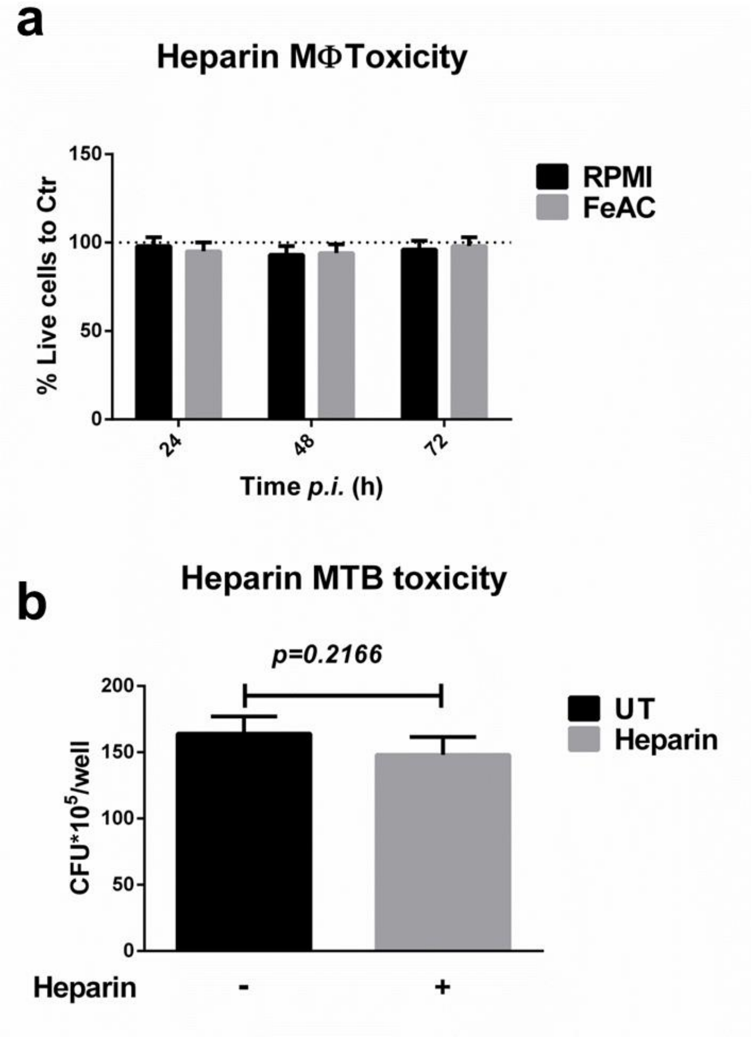

Fig S7.1: **Heparin has no direct impact on host cell or bacteria viability**. a) THP-1 cell viability after heparin (50µg/ml) treatment relative to untreated control. b) Viability of Mtb bacilli after 72 hours treatment with heparin (50µg/ml) in C-RPMI without macrophages. Data from two independent experiments.

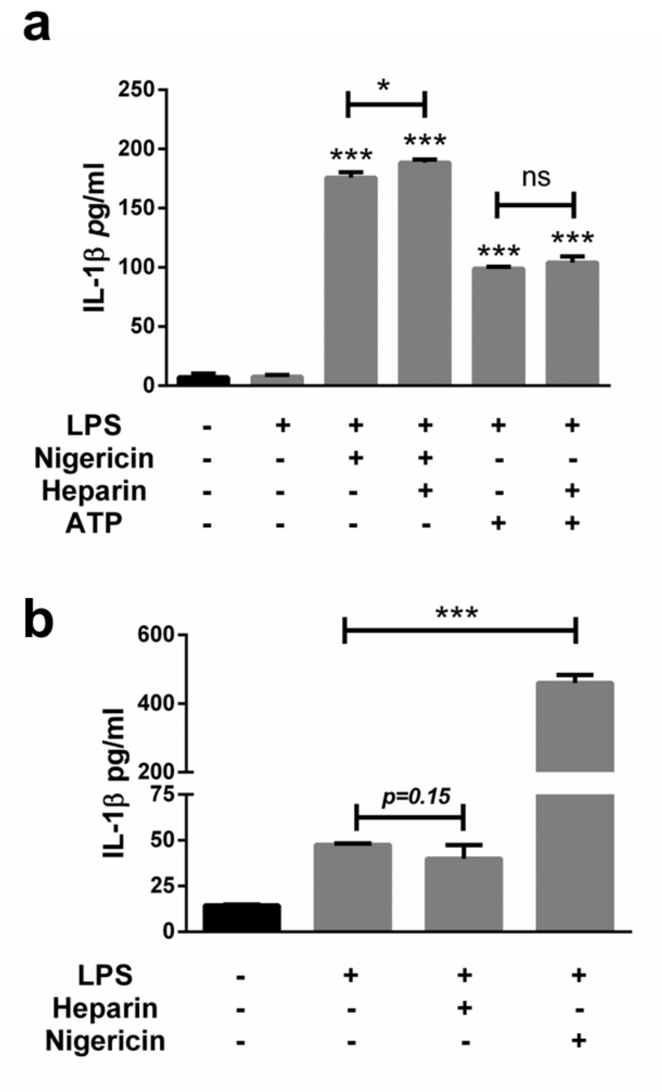

Fig S7.2: **Heparin induces NLRP3-mediated IL-1β secretion by macrophages.** a) IL-1β secretion in culture supernatants of macrophages primed with LPS overnight and treated with nigericin or ATP for inflammasome activation, with or without heparin. b) IL-1β secretion in culture supernatants of macrophages primed with LPS and treated with heparin or nigericin for inflammasome activation. Data from three independent experiments. **P<0.05, ***p<0.001*

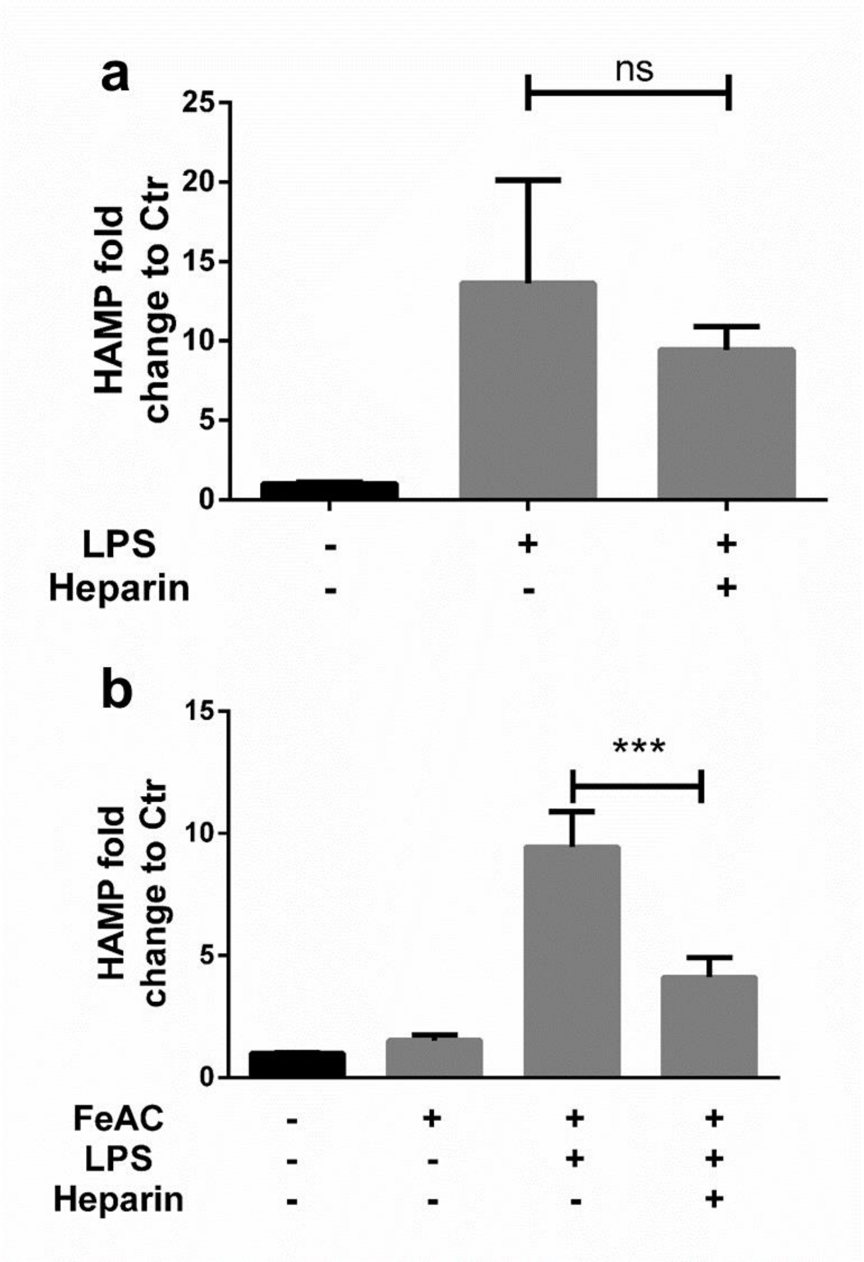

Fig S7.3: **Heparin inhibits LPS-mediated hepcidin expression in iron supplemented media.** Hepcidin expression in heparin-treated macrophages 24 hours after LPS stimulation in normal RPMI media (a) or iron supplemented media (b) measured by qRT-PCR. Data from three independent experiments. ****$p<0.001$*

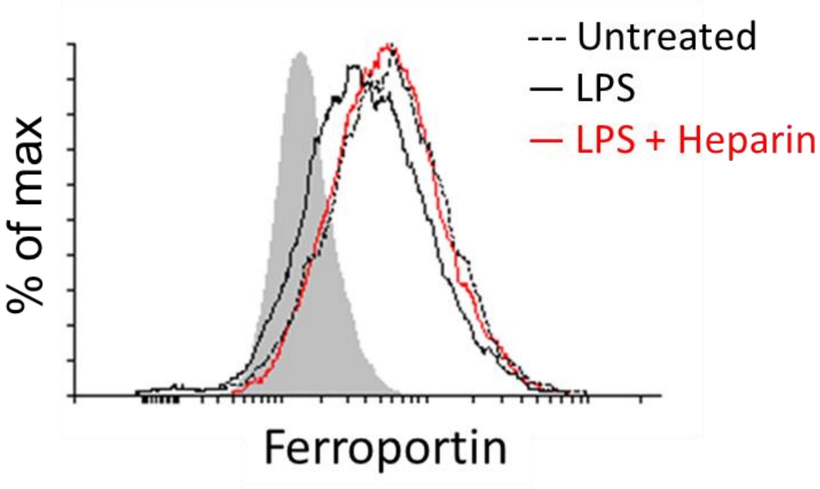

Fig S7.4: **Heparin increases ferroportin surface expression in LPS stimulated THP1 macrophages** THP-1 macrophages differentiated and treated with LPS as described in the material and methods, stained for surface ferroportin and analyzed by flow cytometry.

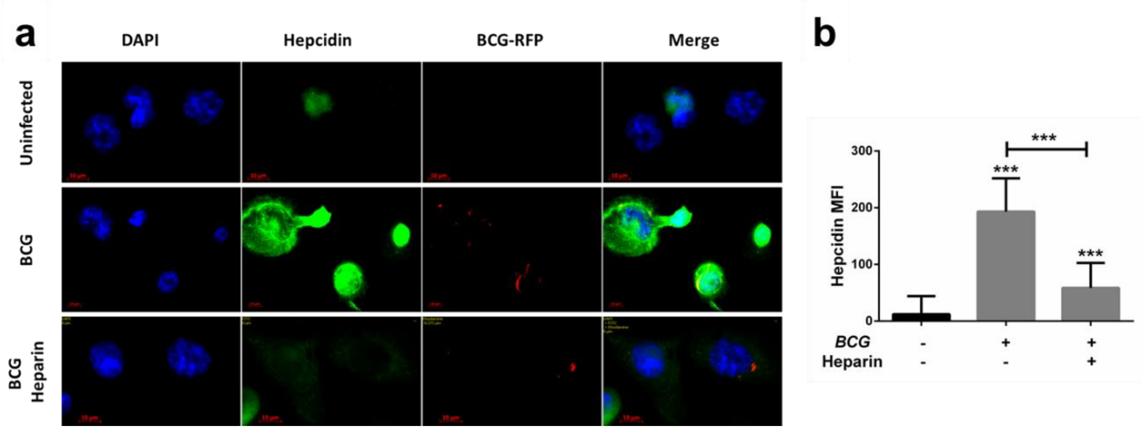

Fig S7.5: **Heparin inhibits BCG-induced hepcidin expression.** a) Hepcidin expression in heparin-treated macrophages 48 hours after BCG infection (63X). b) Pixel MFI/cell from a minimum of 20 cells in three different fields represented in A from three independent experiments.

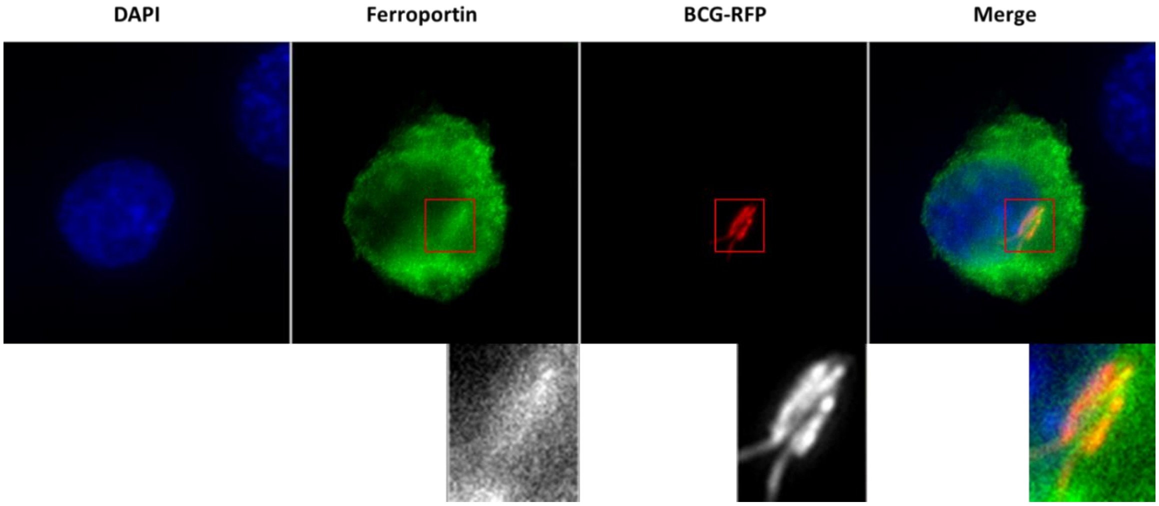

Fig S7.6**: BCG and ferroportin colocalization.** Ferroportin expression in RFP-BCG infected macrophages (100X). THP-1 macrophages differentiated and infected with RFP-BCG as described the material and methods and stained for ferroportin.

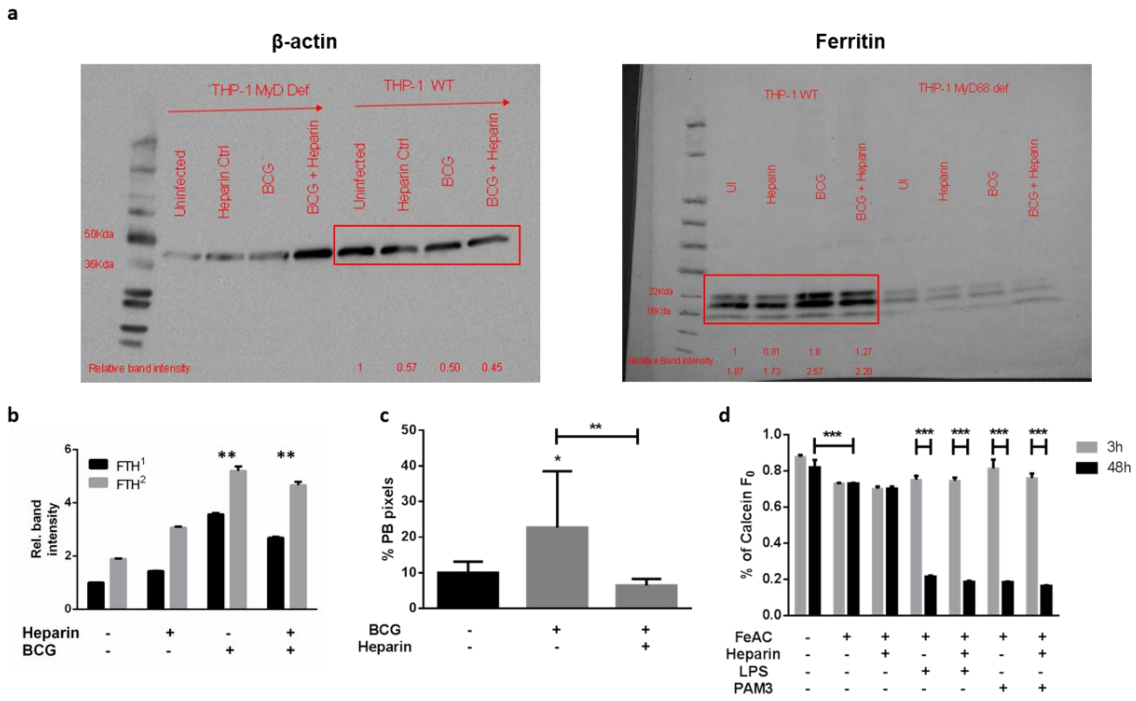

Fig S7.7: **Mycobacterial infection has no impact on the intracellular labile iron pool of macrophages.** a) Ferritin (left) and loading control β-actin (right) expression in heparin-treated THP-1 macrophages 48 hours after BCG infection. b) Relative Ferritin bands (upper FTH^1, lower FTH^2) intensity normalized to loading control as shown in A. c) Percentage of Prussian blue pixels area to total stained pixel surface area in heparin-treated macrophages 48 hours after BCG infection. d) Labile iron levels in heparin-treated macrophages three and 48 hours after LPS or Pam3CSK4 stimulation. A and B data is representative of three independent experiments. C and D data from three independent experiments. **p<0.05, **p<0.01, ***p<0.001*

CHAPTER 8

HEPCIDIN INHIBITION LIMITS *MYCOBACTERIUM TUBERCULOSIS* INTRACELLULAR REPLICATION IN HUMAN MACROPHAGES

Macrophage intracellular iron levels greatly influence bacterial replication during infection with siderophilic bacteria (chapter 6). Iron chelation therapy or increased iron export significantly limit intracellular bacterial burden during infection with *Salmonella sp.*, *Listeria* sp. or *Mycobacteria* sp.[1–5]. Contrastingly, hepcidin secretion with concomitant decreased iron export greatly promote intracellular replication of these same siderophilic pathogens. Here we evaluate the impact of hepcidin blocking during *Mycobacterium tuberculosis* infection and propose hepcidin inhibition as an effective host-directed therapy during this bacterial infection.

We previously showed that *M. bovis BCG* infection greatly promotes intracellular iron sequestration in human macrophages through hepcidin induction and ferroportin downregulation (Chapter 3). To determine if *M. tuberculosis* infection also modulates the expression of the iron-regulating proteins hepcidin and ferroportin, we measured the transcriptional expression of the responsible genes in human THP-1 macrophages after infection. Infection with BCG or *M. tuberculosis* significantly upregulates hepcidin expression and greatly downregulates ferroportin expression favoring intracellular iron sequestration in THP-1 macrophages (Fig 1 A and B). Likewise, hepcidin levels in medium supernatant of THP-1 macrophages are significantly increased immediately after

M. tuberculosis infection and peak 48 hours post infection (Fig 1C). Ferroportin is the only known iron exporter in mammals expressed on the surface of hepatocytes, enterocytes and macrophages[6]. Hepcidin binds to ferroportin leading to its internalization and degradation[7]. To assess the impact of *M. tuberculosis* infection on iron export, we measured ferroportin surface expression in THP-1 macrophages by immunofluorescence staining[8]. As expected, and consistent with qRT-PCR data, 48 hours after *M. tuberculosis* infection surface ferroportin levels are greatly decreased compared to uninfected controls (Fig 1D).

Despite being first described as an antimicrobial peptide against extracellular bacteria, recent reports indicate that during infection with intracellular pathogens such as HIV or *Salmonella enterica*, hepcidin induction increases pathogen replication[9–11]. Contrastingly, ferroportin overexpression and iron export significantly limit intracellular bacterial replication[12]. To evaluate the role of hepcidin in *M. tuberculosis* infection we silenced hepcidin in the THP-1 cell line by transduction with shRNA lentiviral particles as previously described (Chapter 4). As hypothesized, hepcidin silencing significantly decreased intracellular bacterial burdens 48 and 72 hours post infection (Fig 2A). Interestingly, when compared to scramble controls (ShScram), intracellular bacterial replication is decreased by more than 50% in the hepcidin knock down macrophages (Fig 2B).

Hepcidin was first described in urine and blood from patients suffering from chronic inflammation by two independent groups [13–15]. Initially identified as a 25 amino acid peptide with structural similarities to other β-defensins[15], hepcidin is now recognized as the major iron-regulatory hormone in mammals with minimal direct microbiocidal activity at physiological concentrations[14,16,17]. Hepcidin is encoded by the gene human antimicrobial peptide (HAMP) and is first transcribed as an 85 amino acid peptide with a 20 amino acid C-terminal signaling sequence and the 65 amino acid propeptide. Hepcidin propeptide (prohepcidin) has no reported function and the mature functional peptide results from furin cleavage of the last C-terminal 25 amino acid sequence (hep25)[18–20]. A hep25 synthetic peptide is commercially available and has been shown to mimic native hepcidin iron regulatory properties[21]. To assess the importance of hepcidin iron regulatory functions during *M. tuberculosis* intracellular replication, we infected hepcidin silenced THP-1 (ShHAMP) cells with *M. tuberculosis* in hep25 supplemented medium (100 ng/ml) as previously described (chapter 7). As hypothesized, supplementation with hep25 synthetic peptide significantly increases intracellular *M. tuberculosis* replication in ShHAMP THP-1 macrophages and rescues intracellular bacterial burden to similar levels of ShScram cells at 24 and 48 hours post infection (Fig 2A and B).

Hepcidin secreted peptide (hep25) is a central player during hemochromatosis and anemia and has been one of the major therapeutic targets for the treatment of these diseases over the past decade[22]. Hepcidin blocking antibodies specifically bind to hepcidin and decrease its function, significantly decreasing hepcidin-mediated hypoferrimia *in vivo*[23]. To assess the impact of hepcidin blocking during *M. tuberculosis* infection, we infected THP-1 macrophages human in the presence of 1 µg/ml of a hepcidin-specific capture antibody (mab2.7). Similar to hepcidin inhibition with ShRNA HAMP, hepcidin blocking with mab2.7 significantly decreases intracellular bacterial burden in human macrophages (Fig 2C). When compared to untreated controls, mab2.7

treated macrophages contain less than 50% the number of intracellular bacilli by 24 hours post infection, and less than 90% by 72 hours post infection (Fig 2D

Mycobacterium tuberculosis can directly downregulate ferroportin expression independently of hepcidin through TLR2 signaling (Chapter 4). Thus, we determined if inhibiting hepcidin function is sufficient to increase ferroportin surface expression in infected macrophages. As hypothesized, ShHAMP THP-1 cells significantly increased surface ferroportin expression after *M. tuberculosis* infection compared to ShScram (Fig 3A). Similarly, hepcidin blocking with mab2.7 also results in increased surface ferroportin expression in *M. tuberculosis* infected macrophages (Fig 3B).

THP-1 cells were established in 1980 and characterized as a suspension monocytic leukemic cell line. Despite the similarities with primary human macrophages, particularly after PMA differentiation into adherent macrophage-like cells, THP-1 cells do not always mimic the response of primary macrophages to stimuli or infection.

To evaluate the impact of Mtb infection in hepcidin expression by human primary macrophages, we collected and isolated peripheral blood mononuclear cells from healthy donors; purified monocytes by plastic adherence and differentiated them into macrophages with GM-CSF for 5 days [24,25]. Uninfected primary macrophages secrete minimal detectable levels of hepcidin in the media supernatants up to 48 hours after differentiation (Fig 4A).

Similar to THP-1 macrophages, *M. tuberculosis* infection significantly induces hepcidin secretion in human primary macrophages 48 hours after infection ($p<0.001$). In THP-1 macrophages hepcidin blocking with a capture antibody (mab2.7) significantly limits *M. tuberculosis* replication up to 72 hours post-infection (Fig 2C and D). In human primary macrophages, mab2.7 also decreases intracellular bacterial load 24 hours post-infection, but not at 48 and 72 hours post-infection (Fig 4B). Surprisingly, unlike with THP-1 cells, we could not observe *M. tuberculosis* replication in primary macrophages and both treated and untreated groups could effectively control and steadily decrease bacterial burden up to 72 hours post-infection. In the future, it will be important to evaluate the impact of *M. tuberculosis* infection and hepcidin blocking in host cell viability and optimize monocyte purification, macrophage differentiation and *M. tuberculosis* infection protocols to further validate the results observed in these preliminary studies.

Altogether, these results uncover hepcidin as an important therapeutic target during *M. tuberculosis* infection. The pronounced impact of hepcidin inhibition with a specific blocking antibody on intracellular bacterial burden *ex vivo* is extremely promising but requires further studies *in vivo* to better assess the efficacy of such host-directed therapies for tuberculosis. Future studies will assess if hepcidin is highly expressed by alveolar macrophages or in human granulomas from *M. tuberculosis* infected individuals which would further support the relevance of this peptide during infection.

Hepcidin inhibition with mab2.7 significantly increases ferroportin surface expression in *M. tuberculosis* infected macrophages suggesting that iron export and the consequent decrease in intracellular iron availability is the major mechanism limiting *M. tuberculosis* replication in human macrophages. In the future, it will be important to measure total intracellular iron levels in macrophages treated with mab2.7, and the impact of this treatment on iron scavenging by intracellular bacilli. *In vivo,* the use of this antibody might be very beneficial in combination with iron chelation therapy, which efficiently decreases extracellular iron availability or other antimycobacterial drugs that directly kill extracellular bacilli.

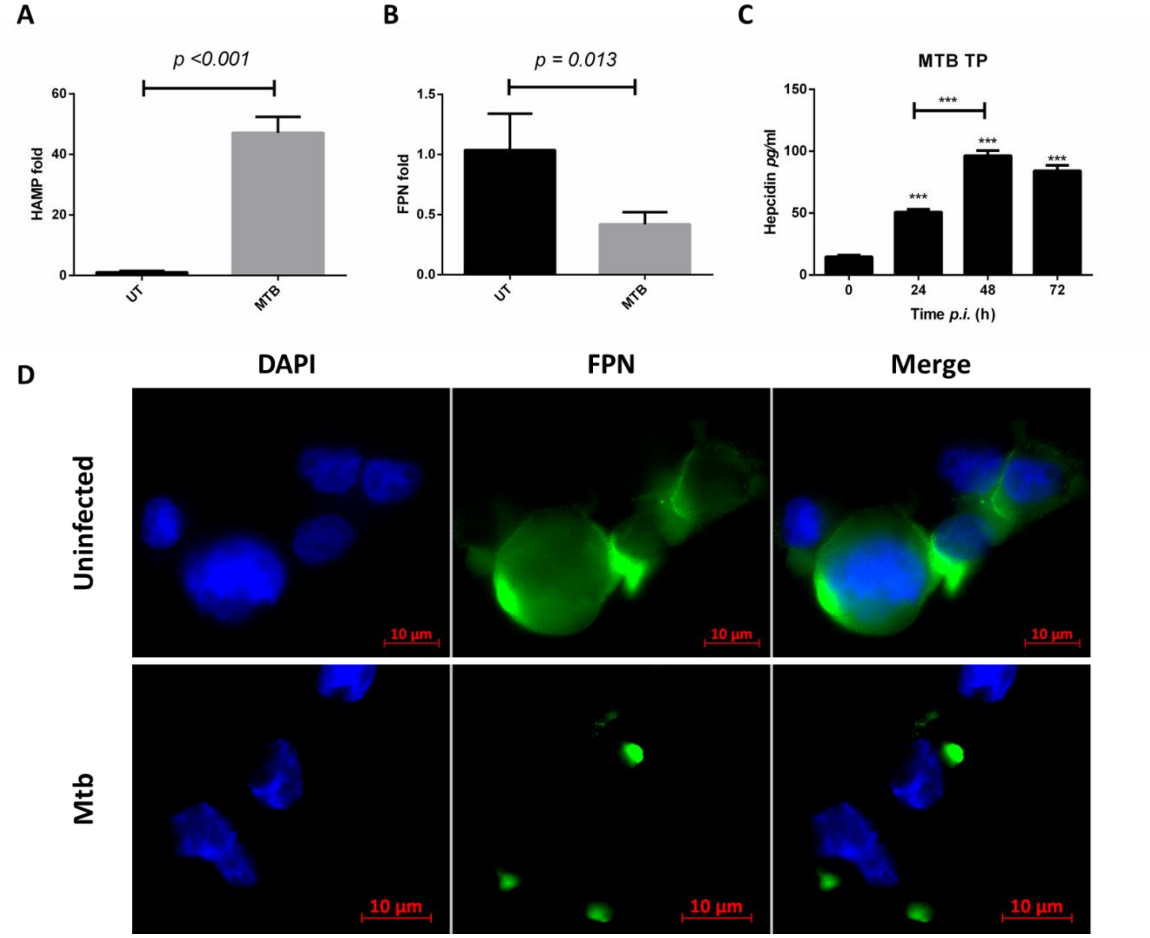

Fig 8.1: ***Mycobacterium tuberculosis* promotes iron sequestration in macrophages through regulation of iron related genes.** Genes encoding A) hepcidin and B) ferroportin transcriptional expression in THP-1 macrophages 24 hours after *M. tuberculosis* infection measured by qRT-PCR. C) Hepcidin secretion in medium from *M. tuberculosis* infected THP-1 macrophages measured by ELISA at different time points after infection. D) Ferroportin surface expression in THP-1 macrophages 48 hours after *M. tuberculosis* infection. A-C data from three independent experiments. D) Representative data from three independent experiments. *** $p<0.001$

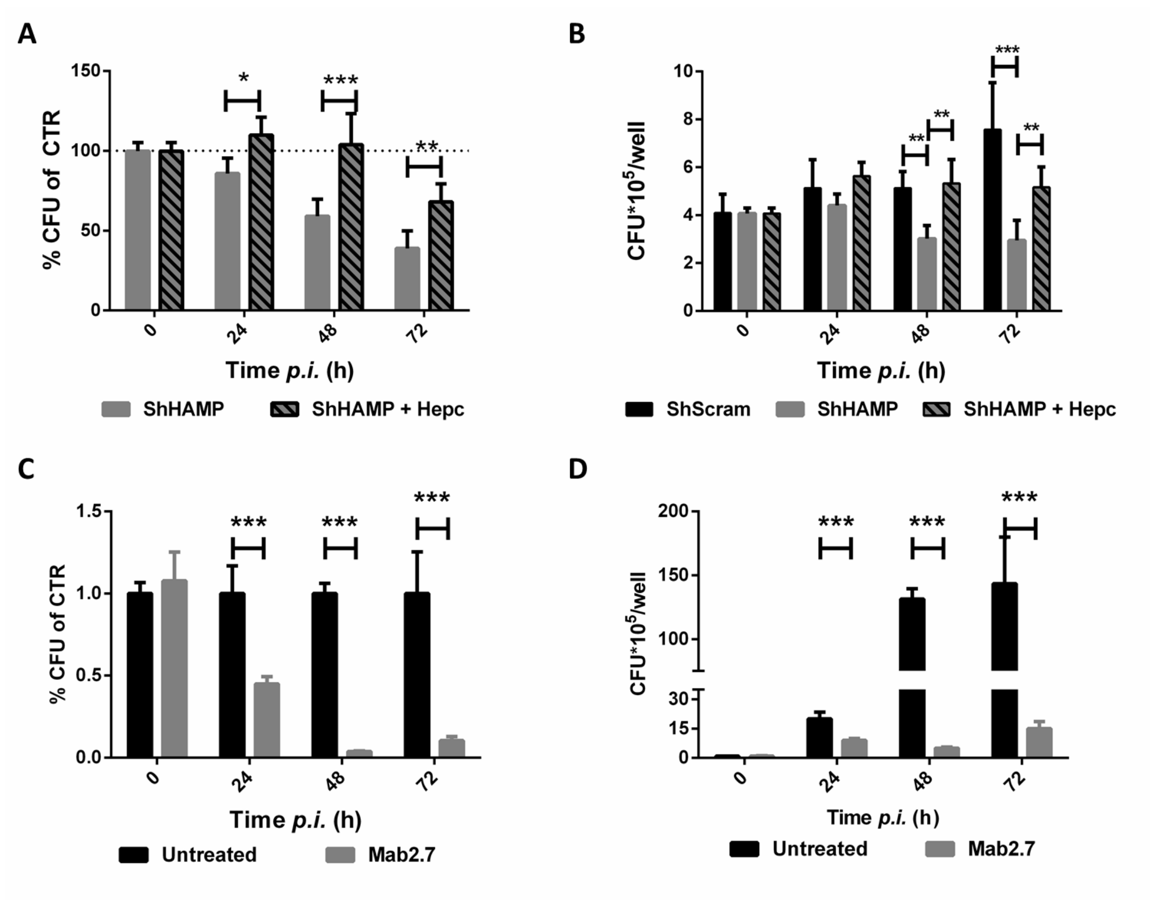

Fig 8.2: **Hepcidin inhibition limits *M. tuberculosis* intracellular replication in THP-1 macrophages.** A) Percentage of intracellular *M. tuberculosis* bacilli in hepcidin-silenced THP-1 macrophages (ShHAMP) to the Scramble control (ShSCRAM) when infected with or without hepcidin synthetic peptide. B) Intracellular bacterial burden in ShHAMP and ShSCRAM infected as in A) were quantified by gentamicin protection assay. C) Percentage of intracellular *M. tuberculosis* bacilli in THP-1 macrophages infected in presence of a hepcidin blocking antibody (mab2.7) compared to respective untreated controls. D) Intracellular bacterial burden in THP-1 macrophages infected in the presence of mab2.7 and respective untreated controls as in C) quantified by gentamicin protection assay. A-D data are from three independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$).

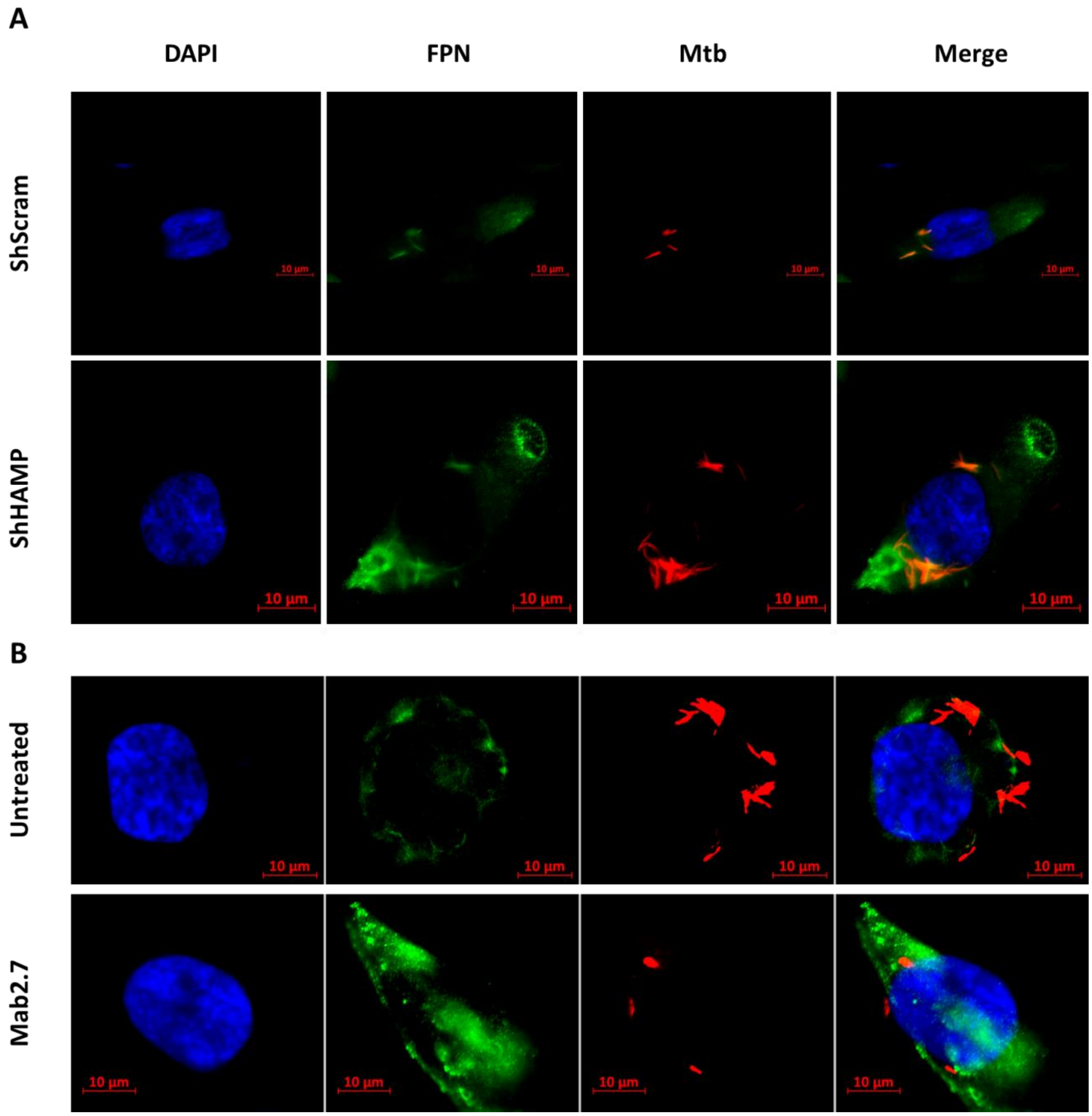

Fig 8.3: **Hepcidin inhibition results in increased surface ferroportin expression in *M. tuberculosis* infected human macrophages.** A) Surface ferroportin in hepcidin-silenced THP-1 macrophages (ShHAMP) and the respective scramble control (ShSCram) 48 hours after *M. tuberculosis* infection. B) Surface ferroportin in THP-1 macrophages 48 hours after *M. tuberculosis* infection with and without hepcidin blocking antibody (mab2.7). Data representative of three independent experiments.

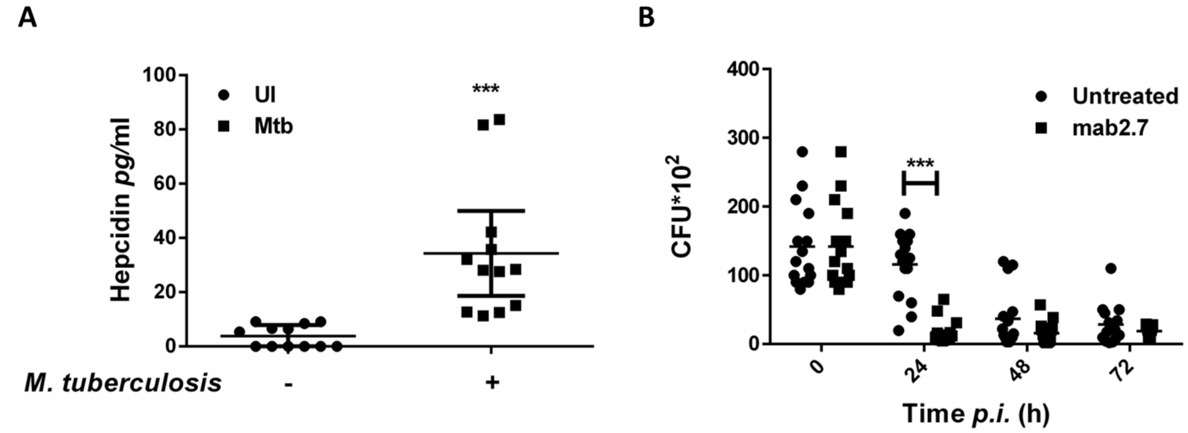

Fig 8.4: **Hepcidin blocking limits *M. tuberculosis* replication in primary human macrophages**, A) Hepcidin secretion in the media supernatants of human primary macrophages 48 hours after *M. tuberculosis* infection. B) Intracellular *M. tuberculosis* burden in human primary macrophages in presence of a hepcidin blocking antibody (mab 2.7). *** p<0.001

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CHAPTER 9

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Notwithstanding the efforts to eradicate it, tuberculosis has again become the leading cause of death due to an infectious disease. Moreover, the rise of multidrug-resistant and extensive-drug resistant cases requires that new therapeutics and a more effective vaccine are developed. In the second chapter of this thesis its shown that bacterial infection and activation of TLR signaling significantly increases intracellular iron sequestration in human macrophages through two redundant and independent mechanisms. There, we demonstrate that during mycobacterial infection, activation of TLR2 signaling directly downregulates ferroportin expression while TLR4 activation induces hepcidin expression and secretion further decreasing ferroportin surface levels and iron export. The similarities between TLR2 and TLR4 signaling pathways make it hard to explain the different molecular pathways leading to intracellular iron sequestration, especially because TLR4-mediated hepcidin expression in macrophages is MyD88 dependent. We still don't understand how TLR2 signaling directly downregulates ferroportin expression, but in the third chapter of this thesis, we show that hepcidin induction in macrophages is mainly dependent on increased ER-stress. Mtb infection and chronic TLR4 activation with LPS has been shown to increase ER-stress in macrophages, however the same is true for TLR2 activation. In the future, it will be important to evaluate the impact of Mtb and *Listeria* infection on ER-stress in macrophages to better understand the molecular pathways resulting in increased hepcidin expression and hepcidin-mediated intracellular iron sequestration in macrophages. Similarly, direct TLR2 and TLR4 activation with purified ligands and well known ER-stress inducers should also help unveil the differences in this molecular cascade.

Iron is a crucial micronutrient for almost all organisms but particularly for siderophilic bacteria. Pathogens must compete with the host for the same iron pool and inefficient iron scavenging severely affects the virulence of pathogenic bacteria. Mtb-mediated hepcidin induction in macrophages significantly increases intracellular iron levels which become available to intracellular bacteria. In the fourth chapter of this thesis we hypothesized that hepcidin inhibition in macrophages would result in decreased intracellular replication during Mtb infection. Heparin has been recently shown to inhibit hepcidin expression in hepatocytes, and in this study we demonstrated that heparin treatment in macrophages could block TLR4-medaited hepcidin expression after LPS treatment or Mtb infection. Following our hypothesis, heparin treatment significantly decreased intracellular bacterial burden of Mtb-infected macrophages compared to untreated controls, and this was dependent of heparin-mediated hepcidin expression. Heparin is a complex glycosaminoglycans with variable sizes and biological functions, best known for its anticoagulant properties. Recent studies seem to indicate that non-anticoagulant oversulfated modified heparins are responsible for hepcidin inhibition in hepatocytes. Future studies will focus on understanding which heparin fractions and modifications inhibit hepcidin expression and decrease intracellular Mtb replication in macrophages. Furthermore, it will be important to understand the role of heparin in Mtb or LPS-induced ER-stress in macrophages.

IFNγ is generally associated with a host-protective immune response during Mtb infection and other intracellular siderophilic bacteria. Increased microbiocidal activity of IFNγ-activated macrophages has been long associated with increased ROS and NO production. However, recently IFNγ was shown to promote iron export in macrophages during *Salmonella* infection resulting in a significant decrease in intracellular bacterial burden. Thus, in the fourth chapter of this thesis, we asked if IFNγ could modulate intracellular iron levels in macrophages to control replication of intracellular siderophilic bacteria such as *Listeria, Salmonella* and *Mycobacteria.* As hypothesized, IFNγ inhibits hepcidin and significantly induces ferroportin expression in macrophages during infection with siderophilic bacteria resulting in decreased iron availability to intracellular pathogens. In this study we unveil a novel mechanism by which IFNγ controls intracellular bacterial replication and exposes iron dysregulation and hepcidin expression as an important factor during both innate and adaptive immunity against intracellular siderophilic bacterial pathogens.

Chapters three and four of this thesis show the pronounced host-protective impact of hepcidin inhibition during tuberculosis. However, IFNγ therapy results in excessive lung pathology *in vivo* and failed in initial clinical trials as a putative host-directed therapy for tuberculosis. Heparin is an approved anticoagulant therapy for post-surgery patients to prevent embolism and thrombosis, but is associated with increased risk of hemorrhage which makes it unsuitable as an effective host-directed therapy for tuberculosis. Thus, in the fifth chapter of this thesis we searched for alternative molecules to inhibit hepcidin function, intracellular iron sequestration and Mtb growth in macrophages. Hepcidin is a secreted protein and its impact on intracellular iron levels is dependent of its interaction with ferroportin in the surface of macrophages. Hepcidin-specific capture antibodies effectively inhibit hepcidin-mediated anemia of chronic diseases and are currently in phase I clinical trials for cancer-related anemia. Therefore, we evaluated if a hepcidin-specific capture antibody could also decrease intracellular mycobacterial replication in macrophages. As hypothesized hepcidin blocking with a specific capture antibody significantly increases ferroportin surface expression and decreases intracellular iron sequestration in macrophages and efficiently limits intracellular mycobacterial replication.

This thesis explores the importance of macrophage iron status in the outcome of disease during Mtb infection. Decreasing intracellular iron sequestration in macrophages greatly limits Mtb replication and other siderophilic intracellular bacteria. In the future it will be interesting to see if hepcidin is highly expressed in the granulomas of infected individuals and if inhibiting hepcidin expression or increasing ferroportin levels *in vivo* decreases Mtb bacterial load in the lungs. Mechanistically it will be important to confirm if alveolar macrophages show increased hepcidin expression and intracellular iron sequestration during Mtb infection, and if this outcome is dependent of Mtb-induced ER-stress as here observed with a human macrophage-like cell line. Altogether, the studies here described show for the first time the importance of macrophage intracellular iron levels for mycobacterial replication and uncover a novel target for host-directed therapeutics in tuberculosis.