Iron Metabolism and the Inflammatory Response

Abstract
Iron (Fe) is essential to almost all organisms, as required by cells to satisfy metabolic needs and accomplish specialized functions. Its ability to exchange electrons between different substrates, however, renders it potentially toxic. Fine tune mechanisms are necessary to maintain Fe homeostasis and, as such, to prevent its participation into the Fenton reaction and generation of oxidative stress. These are particularly important in the context of inflammation/infection, where restricting Fe availability to invading pathogens is one, if not, the main host defense strategy against microbial growth. The ability of Fe to modulate several aspects of the immune response is associated with a number of “costs” and “benefits”, some of which have been described in this review.

Keywords: iron; inflammation; infection; immunity; iron metabolism

Introduction
Iron (Fe) is a very abundant atom, representing almost 5% of the earth’s crust. Most forms of Fe are insoluble, i.e. not biologically useful. Thus, essential is the role played by proteins, e.g. ferroxidases and ferrireductases, which are capable to convert Fe into a more soluble form and increase its accessibility. The poor solubility of Fe, which in neutral solutions corresponds to $10^{-18}$ M for the most abundant forms Fe$^3^+$ and to $10^{-2}$ M for most ferrous salts Fe$^{2+}$, could be seen as part of an entire strategy to avoid Fe toxicity (1). In fact, if from one side this metal is essential to almost all organisms, on the other, it becomes toxic when accumulating above certain thresholds. The ability of Fe to stably interconvert between the most common oxidative forms Fe$^{2+}$ and Fe$^{3+}$ favors its participation into the Fenton reaction and the generation of highly reactive hydroxyl radicals (2). These subsequently damage DNA, lipids and proteins, causing cells to undergo Fe-mediated oxidative stress and programmed cell death. Thus, maintaining Fe homeostasis is a necessary step for the correct cell functioning and to prevent Fe-mediated tissue damage (3).

Iron Absorption
In humans, dietary Fe absorption is strictly regulated and influenced by the type of food ingested. While compounds, e.g. phytates, act as inhibitors of Fe uptake, others like vitamin C increase Fe entry and facilitate its reduction to Fe$^{2+}$ (4). Dietary Fe absorption varies between 1 and 2 mg. This represents only a small fraction of its daily requirement, which accounts for approximately 30 mg. Both inorganic and organic Fe can be absorbed. Different forms of this latter are found in Fe-binding compounds e.g. lactoferrin and plant phytotransferrin. These are particularly important in individuals fed with poor Fe diets or during the neonatal period (5-7). The lower the levels of Fe in the body, the higher its intestinal uptake. The expression of the divalent metal transporter 1 (DMT1) (8) and of the duodenal membrane associated cytochrome b ferrireductase (DcytB) on enterocytes enables the reduction and
subsequent absorption of inorganic Fe (9,10). The ability of enterocytes to directly internalize nanoparticulate nonheme Fe allows the potential endocytosis of the whole ferritin-Fe core. This derives from plants and meat-based dietary sources, and is then dissolved into intracellular vesicles by lysosomal degradation (11). The absorption of organic heme/Fe, from animal diet, occurs through the expression of the heme carrier protein-1 on mature villus enterocytes, in the duodenum and proximal jejunum (12). Once internalized, heme/Fe is subsequently degraded by the actions of the heme catabolizing enzymes, heme oxygenases (HO-1 and HO-2). This generates carbon monoxide (CO), bilirubin, and Fe$^{2+}$ (13,14), which according to cellular demands is either stored into ferritin nanocages (15,16) or directed to the mitochondria, where used for heme biosynthesis and/or iron–sulfur (Fe–S) clusters biogenesis (3). To prevent the formation of Fe-mediated reactive oxygen species (ROS), excess Fe is then exported from cells, through the action of the Fe efflux pump ferroportin (FPN), which is the only Fe exporter described so far (17,18). The cooperation of membrane bound ferroxidases, e.g. the multicopper ferroxidase hephaestin (19) or the serum copper cooperation of membrane bound ferroxidase, e.g. the same protein, are known to regulate Fe metabolism (28). An example is provided by the transcription of FPN, which is triggered by a Fe-dependent and Fe-independent mechanism (29). Micro-RNAs (miRs), i.e. noncoding RNAs, formed of 19 to 25 nucleotides, were also shown to influence Fe homeostasis, since capable to negatively regulate gene expression by inhibiting and/or reducing the translation/half-life of a great number of miRNAs. While miR-210 targets TR1 and modulates Fe acquisition (30), Fe storage and utilization are controlled by miR-200b, which post-transcriptionally reduces FTH expression (31). Interestingly, a regulatory network between FTH and miRs levels has been observed (32). Hence, FTH silencing is associated with significant modifications in the expression profile of genes involved in cell death, survival, hematological system development and function, which are regulated by miR-125b (33). Also the release of Fe is controlled by miRs. The action of miR-485-3p on FPN represses its expression, thus increasing the levels of intracellular Fe storage (34).

At systemic level, Fe homeostasis is maintained by the expression of specific Fe sensors, produced by the liver. Under homeostatic conditions, i.e. when the level of body Fe is sufficient, complexes formed between the hemochromatosis gene (HFE) and TIR-1 prevents Fe uptake (35). When the level of Fe-Tf increases, the dissociation of HFE-TfR1 releases TfR1, allowing HFE to associate with TFR2, matriptase-2, and hemosiderin, forming a multiprotein complex. This activates the BMP–SMAD signaling pathway, which subsequently promotes the transcription of the liver Fe-regulatory hormone, hepcidin (36,37). When released into circulation, hepcidin binds to the extracellular portion of FPN on the basolateral membrane of the cells, promoting its internalization and subsequent degradation (18). This results in intracellular Fe retention, inhibition of intestinal Fe absorption and Fe recycling from hemophagocytic macrophages, which prevents further release of Fe into circulation. The ability of hepcidin to modulate the expression of Fe-importers, e.g. DMT1, allows understanding the
Iron in Inflammation and Infection

Inflammation and infection are characterized by changes in Fe metabolism, a cross-talk facilitated by the presence of binding sites for proinflammatory cytokines in the promoter of genes regulating Fe homeostasis (41). One of the first responses triggered by inflammatory conditions is an increased level of hepcidin, which responds to the release of IL-6 and IL-1 (42,43). Closing the entry doors for Fe into circulation, hepcidin plays a major role in restricting Fe availability to pathogens and preventing their proliferation. It is, therefore, one of the main defense mechanisms against infection. Defects in the control of Fe homeostasis, as occurring in patients with HH, where mutations in hepcidin does not prevent further Fe absorption, result in an enhanced susceptibility to infections (44). The opposite is observed in anemic conditions (45) and allows understanding the importance of a subject that is highly investigated in recent years, i.e. nutritional immunity (4,46).

When referring to Fe metabolism, several differences have been observed in infections caused by intracellular or extracellular pathogens (47). The proliferation of microbes within host cells entirely relies on intracellular resources. Hence, it is microbes’ interest to keep cells alive as long as possible and to inhibit the use of intracellular Fe for the engagement of antimicrobial mechanisms that lead to pathogen clearance (44). Thus, the virulence of intracellular pathogens, e.g. *Mycobacterium tuberculosis*, does not increase in the absence of hepcidin and/or in Fe-loaded conditions (48). Nevertheless, for pathogens to exploit nutrients and proliferate feeding on intracellular Fe stores without undergoing Fe cytotoxicity, they need to metabolically adapt to host environment (49). This implies the development of mechanisms that are capable to provide a survival advantage over immune cells, e.g. reducing FPN expression with the aim to turn host Fe into pathogens’ nutrient source. This effect also renders immune cells refractory to the release of proinflammatory cytokines and/or the production of humoral antibodies (50). In keeping with this notion, FPN overexpression was shown to inhibit the growth of intracellular pathogens, e.g. *M. tuberculosis* and *Salmonella enterica*, within macrophages (51), presumably via a mechanism that cause Fe nutrient deprivation. Inhibition of lysosomal fusion and destruction of phagosomal membranes are used by microbes to avoid the action of catalytic enzymes. Since this impacts the degradation of Fe-storing proteins, e.g. ferritin, it results in an increased availability of Fe to intracellular pathogens (52). Ferritin degradation occurs through a protein known as NCOA4, located on autophagosomes’ surface. When the levels of intracellular Fe are high, the expression of NCOA4 is low, which results in the degradation of fewer ferritin molecules. The opposite occurs in the presence of low levels of Fe, which increase NCOA4 and trigger what is referred to as ferritinophagy, a process that leads to higher amount of intracellular Fe (53). Disruption of NCOA4-mediated ferritinophagy is deleterious to Fe-dependent processes, e.g. heme and hemoglobin synthesis during erythropoiesis (54,55). The abundance of NCOA4 controls ferritinophagy and, therefore, understanding the molecular mechanisms underlying its regulation will be essential to unravel the processes of subsequent Fe utilization. However, the difficulty of studying the pool of NCOA4 targeting ferritin to autophagosomes is a major limitation, since this is also degraded within the lysosomes (55). Nevertheless, the involvement of lysosomal dysfunction in the development of a variety of metabolic diseases (56) suggest that NCOA4-induced ferritinophagy might play a crucial role in pathologies associated with disruption of Fe homeostasis. This hypothesis is also supported by the ability of Fe to modulate the function of this organelle. However, it remains still to be established whether, during infection, lysosomal dysfunction is directly caused by pathogens, which might take advantages of potential defects in NCOA4-induced ferritinophagy for their own survival. This hypothesis is reinforced by the observation that microbes might use, as nutrient, the pool of reactive Fe (57), which accumulates in malfunctioning lysosomes, to proliferate, and establish the infection. Whether inhibition of NCOA4-driven ferritin degradation might prevent the programmed cell death caused by intracellular Fe accumulation might also be the case, and therefore this would provide further protection to invading microbes. Different strategies might be engaged to limit Fe accessibility to intracellular pathogens, including an increased expression of the DMT Nramp 1 (58). When recruited to late endosomal membranes of phagocytic cells, the ability of Nramp 1 to transfer Fe to pathogen-containing phagosomes results in pathogen’s death, due to a catalyst effect of ROS formation. The involvement of Nramp 1 to export excess Fe from phagosomes was also observed, thus indicating its importance in depriving pathogens from their nutrient (59). The protective role of Nramp 1 in dictating the outcome of intracellular infections has been well established, as observed when preventing the proliferation of microbes such as *Mycobacterium spp.*, *Salmonella spp.*, and *Leishmania spp* (59,60). Interestingly, the different strategies engaged by intracellular and extracellular pathogens to ensure their growth rely on a distinct ability to manipulate genes controlling Fe metabolism. Thus, according to the type of infection, the result of the host-pathogen competition for Fe provides survival advantage to one or the other (61). Microbes Fe uptake might also occur through ferritin, used as nutrient source by pathogens like *M. tuberculosis*, which take advantage of host increased ferritin expression. Nevertheless, the salutary properties of ferritin in preventing a number of infectious diseases, caused by extracellular pathogens, have been well established (47,62). Higher ferritin levels are observed upon infection and result from intracellular Fe accumulation.
due to hepcidin-mediated FPN degradation. This leads also to an increased intracellular labile iron pool, which usually accounts between 3% and 5% (6–16 μM) of the total Fe content (63). To prevent the deleterious effects of this accumulation, Fe is then transferred to specialized Fe-neutralizing and storing ferritin nanocages (64). In parenchyma tissue, the ferroxidase activity of Ferritin H chain, which converts Fe into an inert metal and then stores it within ferritin subunits, was shown to afford protection against oxidative stress-mediated programmed cell death (65). The protective effects of FTH, conferring metabolic adaptation to Fe overload, were demonstrated in hemolytic and nonhemolytic diseases. In severe forms of malaria, its antioxidant properties were shown to provide disease tolerance to infection, a salutary mechanism that, limiting Fe accessibility to microbes, prevents tissue damage irrespectively from pathogen burden (62,66).

To ensure their survival, new mechanisms of adaptation are acquired by pathogens even in the presence of Fe restricted conditions (46). As an example, in Vibriocholerae infections Fe is accessed also when complexed to Tf. Under normal conditions, Fe-Tf concentration varies, in plasma, between 10% and 30%, while in pathological conditions, e.g. HH, it reaches almost 100%. Hence, patients suffering from Fe overload present exacerbated susceptibility to V. cholerae when compared to controls. The inability of Tf to sequester further Fe into circulation and exert antimicrobial properties also accounts for the lethality associated with Vibrioulnificus infection, which rarely proliferates in healthy individuals but in patients with HH (67). The production of small Fe-scavenging molecules, known as siderophores, is widely employed by pathogens to sequester Fe and counteracted by the host upregulation of lipocalin-2, which is released, in particular, by neutrophils (68). However, microbes, e.g. Hemophilus influenzae or Trichomonas vaginalis, develop also other strategies to acquire Fe. These include the direct uptake of this metal from the heme contained within red blood cells. Other pathogens, e.g. Staphylococcus epididymitis, access Fe from nontransferrin-bound iron (NTBI). NTBI appears in circulation only when the Fe-binding capacity of Tf and other chaperones e.g. citrate, is exceeded (69). Thus, the higher the NTBI levels, the higher the susceptibility to infection, which has also been demonstrated by the increased pathogenicity observed when animals are exposed to excess Fe (67). This further confirms that one of the main defense provided by hepcidin upregulation is the inhibition of NTBI (70,71).

No NTBI available to pathogen is detected in the blood stream of anemic patients, where the levels of hepcidin are quite low. Although anemia is one of the major health problems worldwide, tropical countries in particular, it is known to confer protection against infections. Therefore, whether anemic conditions might be developed to prevent pathogens invasion, especially in areas where vector-born diseases, e.g. malaria, are a major threat, could be the case (4). This theory is supported by the severe complications observed when young children, living in malaria endemic areas, were treated with Fe supplementation. An exacerbated mortality rate was observed, which was caused by the enhanced proliferation of malaria parasites and pathogens that until then were kept under control (45,72). This was confirmed by the notion that the replication of pathogenic bacteria in the blood of individuals under Fe supplementation dramatically increases few hours after consumption. In anemic patients, all Fe is safely bound to Tf, which represents the rate-limiting factor for pathogens growth. However, increased NTBI levels have been observed in response to Fe supplementation, since the concentration of this metal were exceeding the chaperone capacity of Tf (71). In endemic malaria regions, Fe supplementation was not beneficial also when administered to pregnant women (45,72). These started to become more susceptible to infections, which result in the development of placental malaria and premature fetus death. Interestingly, a positive correlation was shown, in mice, between Fe accumulation in the placenta and fetal death (73). Thus, better parameters than circulating Fe are being investigated to assess the feasibility of Fe administration in endemic areas. These include measuring the levels of hepcidin, which role in diminishing Fe into circulation is also used by Plasmodium in the attempt to prevent the establishment of concomitant infections during the initial phases of the disease. In mice, Fe chelation therapy was shown to afford protection against the toxicity of this metal, which mostly occurs during the hemolytic phase of malaria (65). However, further studies would be required to assess the impact of this treatment on the disease and associated anemia, which would allow evaluating the costs and benefits of this approach towards specific infections. To note that the therapeutic treatment of Fe depletion was proven to be effective in inhibiting intracellular infections, since limiting pathogens growth and restoring an appropriate immune response (74).

Iron Metabolism and the Inflammatory Response

As for other compartments, immune cells require Fe for their correct functioning, i.e. for proliferation, differentiation, and activation processes. However, the ability of Fe to alter immune function confers this metal immunoregulatory properties (Fig. 1), which are still poorly studied but known to influence the response against specific pathogens (50,75). One of the first mechanisms by which immune cells exert antimicrobial functions relies on an exacerbated ROS production and oxidative stress generation that ultimately kill microbes. Nevertheless, although fueled by Fe, this phenomenon is impaired in many infections. Increased levels of Fe inside immune cells reduce their capacity to kill pathogens, as shown for Salmonella (58). When Fe is removed, microbial proliferation is inhibited, as also demonstrated by the reduced growth of Candida albicans, upon apo-Tf administration. This also reestablishes the fungicidal activity of macrophages, which then become more prone to kill the pathogen (76). The inhibitory effect of Fe on these phagocytic cells also involves the suppression of pro-inflammatory cytokines release. A reduced production of interferon-γ is observed when macrophages are
exposed to Fe overload, an effect accompanied by an increased release of anti-inflammatory molecules, e.g. IL-4 and IL-10 (58). This Fe-driven impaired immune function also occurs in patients suffering from HH, who are more sensitive to infections (77). The same, in individuals affected by noninherited pathologies characterized by hepatic Fe accumulation, who are less responsive to interferon-α therapy (78). The expression of TfR1 and FPN on dendritic cells was shown to be Fe-regulated as well as their ability to differentiate and acquire a mature phenotype. Effects on myeloid cells have also been reported and Fe was described as capable to: (i) inhibit antigen presentation in a time- and dose-dependent manner (79), (ii) impair antigen processing and presentation to T and B cells (80,81), (iii) enhance the oxidative stress these cells might suffer from (50), and (iv) damage the lipids responsible to mediate proteins interactions (79). Lymphocyte proliferation is lower in Fe-loading conditions and accompanied by a decreased ability to engage mechanisms of DNA repair. This leads to an increased number of DNA breaks, as indicated by the chromosomal damage reported in patients with HH (82). Low numbers of peripheral cytotoxic CD8+ T cells and increased CD4/CD8 ratios were detected in presence of Fe-overload as well as in the peripheral blood of patients with HH. It is interesting to notice that abnormally high CD4/CD8 ratios correlate with an increased disease severity, while asymptomatic HH forms present normal levels (83). This allowed revealing a direct correlation between increased Fe accumulation and the relative expansion of the two main T-cell subsets. While this phenomenon is not reverted by Fe removal, it was shown linked to a decreased cell surface expression of major histocompatibility complex-class I molecules, responsible for the impaired expansion of CD8+ T cells. To note that HFE encodes major histocompatibility complex-class I-like protein, which is mutated in approximately 85% of patients with HH (84). This results in a low number of total lymphocyte counts and higher proliferation of CD4+ T cells (77). Therefore, the Fe mobilized with phlebotomy, which is associated with a faster re-entry of Fe into the Tf pool, correlates with the number of CD8+ but not CD4+ T cells, as observed also in response to Fe chelation therapy. Noteworthy, the up-regulation of TfRs is crucial for lymphocytes’ functions, since their development and activation strictly depend on an increased intracellular Fe uptake. The complete arrest of T-cell differentiation in the absence of TfR1 strongly supports this notion (85) and aids to clarify the reduced levels of CD4+ T cells and decreased CD4/CD8 ratio observed in Fe-deficient conditions (86). While these effects are reverted by Fe supplementation, it is well established that the dysregulation of Fe absorption and tissue Fe accumulation correlate to an impaired number of lymphocytes, which result in increased circulating CD8 T suppressor cells and immunoglobulin-producing B cells (87). To notice that Tf-independent pathways of Fe acquisition have been also shown
involved in supporting B lymphocytes’ growth, as occurring in leukemia cells (88). It is interesting to point out that different forms of Fe are capable to affect lymphocyte proliferation and their subset ratio in a distinct manner. However, the levels of CD8 are always reduced, thus revealing a defective immunoregulatory control (89). This notion is supported by the low amount of nitric oxide and IL-2 secreting lymphocytes, observed in response to increased Fe concentration, which results in poor allo-specific cytotoxic responses (90). When exposed to Fe, the altered expression of T-lymphoid cell surface markers and its ability to influence the expansion of different T-cell subsets was shown to significantly impair immune cells functions. High levels of Fe also affect memory CD4+ T lymphocytes, since decreasing the cloning efficiency of precursor T cells and interfering with antigen-specific lymphocyte responses (91). The impairment caused by Fe on the immune system does not spare natural killer cells, as demonstrated by their reduced lysing efficiency, when Fe is above certain thresholds. This effect is then reverted by Fe chelation therapy (92). The immunoregulatory role of Fe also affects complement activation, this being an essential component of the inflammatory response. Perturbed complement activities were observed in patients suffering from Fe overload, where the engagement of the C3 signaling pathway is particularly compromised, as the processes of antigen phagocytosis and its safe elimination (50,93).

Potential Implications of the Immunoregulatory Properties of Iron

Besides impairing immune function and modulating the susceptibility to infections, preliminary data from our laboratory suggest that disruption of Fe homeostasis in immune cells might have long-term and deleterious implications. The generation of Fe-loaded cells, occurring as a defense strategy against the invasion of extracellular pathogens or simply due to the subacute inflammation characterizing the aging process, might predispose to the development of a variety of pathologic conditions (Fig. 2). The severity of what we might refer to as “side effects” also depends on the ability of these cells to infiltrate the parenchyma tissue. By turning proinflammatory (94), these Fe-loaded immune cells increase the expression of adhesion molecules, which facilitate their migration into the affected organs and contribute to raise the level of tissue Fe. Thus, crucial is the role played by the expression of proteins regulating Fe homeostasis, e.g. ferritin, which importance in parenchyma tissue has already been demonstrated in the context of malaria infection (65). Particularly affected by this potential infiltration are organs with a low regeneration
capacity, which are more prone to undergo oxidative damage and Fe-mediated programmed cell death. Thus, the disruption of Fe homeostasis in immune cells might be considered as a predisposing factor, capable to prime the development of pathological conditions. This notion is also supported by the levels of proteins regulating Fe metabolism that are measured into circulation, since many act as biomarkers for the prognosis of a variety of diseases. The synovial changes occurring upon the chronic disruption of Fe homeostasis is one example of Fe-driven immune system impairment. This notion is also supported by our current investigations in an experimental mouse model of arthritis and the manifestation of this affection by patients suffering from HH. Whether changes in Fe levels are capable to disrupt the cell-to-cell communication between immune system and bone parenchyma, which is critical for the progression of this chronic disease, is currently under study (unpublished data). The disruption of Fe homeostasis, occurring as cause or consequence of a defective immune system, also underlies tumor development and its rapid progression. An increased susceptibility to tumors is indeed observed in patients with HH (95,96). The role of Fe in cancer has been amply documented, as facilitating the proliferation of cells that require higher nutritional and energetic supplies, when compared to normal controls. Thus, an increased Fe uptake has been observed in different types of cancer, ranging from myeloma to breast and colorectal (97). Whether the molecular mechanisms at the basis of these occurrences implies an impaired antigen-specific immune response, which is important in the first phases of tumor development, as well as a compromised activation of cytotoxic T cells or concomitant increase in suppressor T cells, caused by Fe, might be the case, since these are processes that significantly contribute to. Similarly, changes in immune Fe homeostasis were shown to compromise cardiac function in patients both with thalassemia and HH. The release of proinflammatory cytokines, which is favored by Fe accumulation, is responsible for an increased cardiomyocytes mass, protein unfolding and heart Fe overload, which subsequently result in dysfunctional systoles and/or diastoles (98,99). Whether Fe-loaded cells are also capable to migrate into the brain and cause neuroinflammation, upon breaching the blood brain barrier, seems to be the case. Their ability to convert immune resident microglia into proinflammatory cells might contribute to neurodegeneration and enhance the severity of neurodegenerative diseases (unpublished data) (100). Whether this effect can be reverted by the administration of Fe chelators targeting specific cell subtypes is object of our investigation.

**Conclusions**

Fe plays an important role in host defense against infection and its ability to modulate the immune response should be further explored. While the benefits of disruption of Fe homeostasis in immune cells rely on restricting Fe availability to pathogen growth, the costs to it associated might imply long-term consequences. Thus, the potential role of Fe as a predisposing immune regulator factor for the development of pathological conditions, characterized by its accumulation, requires additional studies.

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