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Iron overload and immunity

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Abstract
Progress in the characterization of genes involved in the control of iron homeostasis in humans and in mice has improved the definition of iron overload and of the cells affected by it. The cell involved in iron overload with the greatest effect on immunity is the macrophage. Intriguing evidence has emerged, however, in the last 12 years indicating that parenchymal iron overload is linked to genes classically associated with the immune system. This review offers an update of the genes and proteins relevant to iron metabolism expressed in cells of the innate immune system, and addresses the question of how this system is affected in clinical situations of iron overload. The relationship between iron and the major cells of adaptive immunity, the T lymphocytes, will also be reviewed. Most studies addressing this last question in humans were performed in the clinical model of Hereditary Hemochromatosis. Data will also be reviewed demonstrating how the disruption of molecules essentially involved in adaptive immune responses result in the spontaneous development of iron overload and how they act as modifiers of iron overload.

Key words: Iron; Iron overload; Innate immunity; Adaptive immunity

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THE TWO IMMUNITIES

The last decade has seen a growing understanding of the numerous functions of the immune system beyond the two classical attributes of adaptive immunity: antigen specificity and memory. The key effector cell of adaptive immunity is the lymphocyte, a cell no longer known from its two principal origins in mammals, the thymus (T) and bone marrow (B), but with several subtypes characterized by different function and cytokine production profiles[1]. “Behind” adaptive immunity lays a complex world of cells and molecules involved in less antigen-specific tasks collectively dedicated to the function of innate immunity. Two competing models have sought to explain innate immunity. One where evolutionary conserved features of pathogens are recognized by pattern recognizing receptors[2] known generally as toll-like-receptors (TLRs). A second model, known as the danger model, is based on the assumption that products from damaged or stressed cells provide a danger signal to the host, thus evoking an innate immunity response[3]. The effector cells of innate immunity are mostly myeloid cells and lymphocytes whose interaction with target cells does not depend on the recognition of the Major Histocompatibility Complex (MHC). A third cell, the dendritic cell (DC), was first described by Steinman and Cohn in 1973[4]. DCs have been described most elegantly as “the nexus for translating signals from innate recognition into cells guiding adaptive immune function”[5].

One hallmark of immune system cells, whether involved in innate or adaptive immunity, is their capacity to circulate and migrate from the blood compartment into lymphoid and non-lymphoid tissues (innate immunity cells) from non-lymphoid into lymphoid tissues (DCs) and from the blood into the lymph through the peripheral lymphoid organs (lymphocytes). The other is their role in protection from pathogen infections. Since early descriptions of immune cell functions in iron overload[6] knowledge of the reciprocal interactions between immune cell responses, intracellular iron load and response to micro-

environmental changes in iron levels has increased considerably. A decisive contribution to this understanding was the clarification of the genes involved in iron homeostasis. Reviews of the functions of such genes and their link to the reticuloendothelial system and immunity have been published recently[7,8]. The present review will focus on the evidence that the cells of the immune system are equipped to modulate iron homeostasis through the expression of several iron related genes and proteins, and how do innate and adaptive immune cells respond in conditions of iron overload. In addition, the reader will be reminded of the evidence indicating that defective immune system models, particularly experimental models, are associated with the spontaneous development of iron overload[9]. The mechanisms underlying what could be called the "reverse" side of this same coin, i.e. immunity and iron overload are less well understood. However, taking into account that practically all cells of the immune system express iron related genes their contribution to systemic iron homeostasis should no longer be ignored.

IRON AND INNATE IMMUNITY

As a critical element of cellular activity, iron plays a pivotal role in the fight for survival between mammalian hosts and their pathogens, each displaying a wide range of mechanisms for controlling iron acquisition and utilization. Micro-organisms have developed a large number of strategies to acquire iron from the environment and to transport the element to sites of incorporation into biologically important molecules[10,11]. On the other side, the host has developed the capacity to modulate cellular iron metabolism, not only for its optimal utilization as a catalyst for the generation of reactive oxygen species acting as strong antimicrobial molecules, but also in order to make iron less available for the micro-organisms[11,12]. A number of genes and proteins primary involved in iron homeostasis, namely in iron binding, transport and storage are now recognised to display related immunological functions. These are summarized in **Table 1**[13-24]. In addition, it is becoming clear that the cells of the innate immune system, as part of a non-specific defense against infection, are equipped to express genes and proteins that can modulate iron homeostasis both at the cellular and the systemic levels (see also Table 1)[25-42]. One central player in this modulation is hepcidin, first described as a liver derived antimicrobial peptide[28], and now well recognised as a key regulator of iron homeostasis and the anaemia of inflammation, at the interface of innate immunity and iron metabolism [43]. Liver-derived hepcidin is strongly induced during infection and inflammation, causing intracellular iron sequestration and decreased plasma iron levels, a process mediated by the inflammatory cytokine cascade, namely macrophage derived IL-6 and IL-1[32,33]. The mechanism underlying intracellular iron sequestration is mediated by the hepcidin-induced internalization and degradation of ferroportin, the only known iron exporter[44]. The reduction in extracellular iron concentrations is believed to limit iron availability to invading microorganisms, thus contributing to host defense. Recently, hepcidin has been shown to be endogenously expressed by innate immune cells, i.e. macrophages and neutrophils, capable of migrating from the blood to a site of infection, constituting a newly recognised component of the local innate immune response to bacterial pathogens [23]. Myeloid endogenous hepcidin mRNA expression in response to bacterial pathogens was shown to be dependent on the specific activation by the toll-like receptor 4 (TLR-4), the key pattern recognition receptor for LPS[45]. This activation also produces down-regulation of the iron exporter ferroportin. Endogenous myeloid hepcidin production is not stimulated by iron, pointing to different pathways of hepcidin activation in response to infection or iron overload[23]. The mechanisms underlying the differential stimulatory effects of infection and iron overload on hepcidin are still not understood. Interestingly, it was recently shown that hepcidin levels in fish also respond both to iron overload and infection, demonstrating the evolutionary conservation of hepcidin's dual function[46].

In addition to the demonstrated role of neutrophils and macrophages on hepcidin gene activation and ferroportin down-modulation, other iron genes and proteins involved in iron transport from the human phagosome into the cytosol also play critical roles in TLR-4 mediated innate immunity. The natural resistance-associated macrophage protein 1 (Nramp1) is expressed in circulating phagocytes, and is recruited from the lysosomal compartment to the phagosome membrane where it functions as an efficient antimicrobial through a mechanism of iron deprivation[22]. Nramp1 mutations in mice are shown to cause susceptibility to infection with various intracellular pathogens including Salmonella, Mycobacterium and Leishmania[21]. Mutations in the human homologue of the gene, NRAMP1, were also shown to strongly affect the susceptibility to tuberculosis [47]. Nramp2, also known as the divalent metal transporter 1 (DMT1) is another important iron transporter in mammalians [48,49] and, as Nramp1, it is also induced by infection with intracellular pathogens, namely Mycobacterium[50]. In addition, it is suggested to play an important role in recycling iron from RBC-containing phagosomes to the cytoplasm[51]. The amount of available iron in the phagosome is also decreased by the entry in macrophages of neutrophil derived lactoferrin (Lf), another well known potent iron chelator found recently to have also a bridging role between innate and adaptive immunity. Both *in vitro* and *in vivo* studies showed that Lf is able to stimulate the proliferation and differentiation of T lymphocytes from their immature precursors into the Th1 or the Th2 phenotypes thus having an immunoregulatory effect on Th1/Th2 activities[15]. Finally, lipocalin 2 has been recently described as a pivotal component of the innate immune system and the acute phase response[52]. Upon infection the toll-like receptors on immune cells stimulate the transcription, translation and secretion of lipocalin 2 which then limits bacterial growth by sequestering the iron-loaded siderophore[24].

INNATE IMMUNITY AND IRON OVERLOAD

If one or all of the above described iron related immune response genes either fail or the cells are overwhelmed by continuous iron overloading such as seen in transfusional iron overload, one consequence is the development of infection. Infections including rare microorganisms[53] are among the major complications in patients with thalassemia, a group of common genetic disorders of hemoglobin synthesis clinically characterized by severe anemia and blood transfusion-dependent iron overload. Besides the well-known risks of blood borne viral infections associated with multiple transfusions, the increased susceptibility of these patients to infection is known to be associated with a wide spectrum of immune abnormalities which are, at least in part, due to the effect of iron overload, including defective chemotaxis and phagocytosis by neutrophils and macrophages and decreased natural killer cell activity[54].

In contrast with the findings in transfusional iron loading, patients with severe iron overload due to Hereditary Hemochromatosis (HH) do not show evidence of increased susceptibility to infections or iron loading of macrophages. HH is a common genetic disorder of iron overload, the majority of HH patients being homozygous for the C282Y mutation in *HFE*, a gene encoding a protein of the Major Histocompatibility Complex class Ⅰ (MHC class Ⅰ). The C282Y mutation disrupts the correct folding of the a3 domain of the protein, interfering with its interaction with b2-microglobulin (b2m) and consequently abolishing the cell surface expression of the molecule[35]. The surface expression of HFE was shown to have a prominent role in the regulation of iron export from macrophages[55,56].

Monocyte/macrophage abnormalities in HH

Anomalies in monocyte/macrophage cells have been consistently described in HH patients, including low TNF-a production by peripheral blood macrophages upon stimulation with lipopolysaccharide[57] and a significant increase in iron regulatory protein (IRP) activity in monocytes[58], an anomaly that was corrected after phlebotomy treatment. Interestingly, subjects with a tissue iron burden similar to HH patients, but due to secondary iron overload have an IRP activity significantly decreased suggesting that the increased IRP activity in iron overload is specific to the HFE related hereditary form of hemochromatosis[58]. In addition, a study that investigated the release of erythrocyte-derived iron from purified human monocytes obtained from controls and HH patients showed that although HH monocytes phagocytosed less than half the number of erythrocytes taken up by control monocytes, they released twice as much iron in the form of LMW-Fe complex than controls[59]. More recently, increased iron content was described in macrophages from HH patients transfected with wt HFE when compared with HH macrophages transfected with an empty vector[56]. These observations are consistent with previously reported observations in macrophage cell lines derived from C282Y mutated HH patients, where *HFE* was shown to loose its ability to inhibit iron release leading to a relative macrophage iron deficiency[55]. Interestingly, Mycobacterium tuberculosis (M.tb) residing within phagosomes of macrophages from HH patients exhibit a profound defect in their ability to acquire iron from exogenous transferrin and lactoferrin relative to M.tb-infected macrophages from normal controls[60]. Moreover, macrophages from HH patients failed to induce Nef-mediated iron and ferritin accumulation upon HIV-1 infection in contrast to macrophages expressing wild-type *HFE*[61], thus suggesting that *HFE* mutated macrophages may be better equipped to protect from HIV-1 infection, compatible with the description of a long survival in a patient with AIDS and hereditary hemochromatosis[62]. One must therefore wonder whether the failure of macrophages from C282Y *HFE* HH patients to hold on to the iron is the expression of a putative selective advantage protecting from infection brought by the appearance and the establishment of such a mutation.

Iron and the dendritic cells

Very little work has addressed the interaction between iron loading and dendritic cell (DC) function. There is, however, recent evidence indicating that, upon endotoxin induced maturation, DCs increase significantly the expression of TfR1 and down regulate expression of the export molecule ferroportin[63], an observation compatible with an earlier finding of Kramer *et al* who reported that DCs generated under iron deprivation conditions were phenotypically undifferentiated and could not stimulate T cells[64].

IRON OVERLOAD AND ADAPTIVE IMMUNITY

The postulate that the immunological system could have a role in monitoring tissue iron toxicity, as part of its surveillance function, was first advanced in 1978, based on studies on lymphocyte traffic and positioning[65-67]. It was implicit in that postulate that the lymphomyeloid system, and its circulating components participate in the recognition and binding of metals as a protective device against metal toxicity, and the preferential use of indispensable metals, such as iron, by bacteria or transformed cells. While a vast number of studies have clarified the reciprocal interactions between myeloid innate immunity cells and iron metabolism, fewer studies addressed this question in lymphocyte populations. Lymphocyte activation and expansion depend on the expression of transferrin receptors, required for DNA synthesis and cell division[18] and both activated and non-activated T lymphocytes synthesize ferritin[19,20]. Lymphocytes could, therefore, act as a “mobile” and easily “mobilizable” iron-storage compartment protecting from iron-mediated toxicity[65]. This hypothesis motivated the study

of lymphocyte function in iron overload[68]. The influence of iron on the expansion of different T-cell subsets was demonstrated both *in vitro*[69] and *in vivo*, namely in patients with thalassemia[70]. Results in this clinical model, however, are difficult to interpret due to the inseparable effects of blood transfusion, splenectomy, iron chelation therapy and infection. Imbalances of the relative proportions of CD4+ and CD8+ T lymphocytes, with abnormally high CD4/CD8 ratios were later reported in HH patients[71,72], a clinical model where iron overload is not complicated by the effects of transfusion, splenectomy or desferrioxamine. Curiously, in one experimental model of iron overload seen in mice generated with targeted Hmox1 mutations, high CD4:CD8 ratios have been seen in the splenic cell populations of older mice aged 50 wk[34]. These mice also exhibited numerous activated CD4+ cells. Hepatic inflammatory cell infiltrates were seen in the mice, a finding similar to that reported by Rodrigues *et al* in aging *Hfe* deficient mice[73].

T lymphocyte abnormalities in HH

Abnormalities in the relative proportions of the two major T lymphocyte subpopulations have been consistently described in HH patients. Reimão and co-workers first described that patients with abnormally high CD4/CD8 ratios displayed a faster re-entry of iron into the serum transferrin pool after intensive phlebotomy treatment than patients without those abnormalities [71]. It was shown later that the amount of iron mobilized by phlebotomy correlated significantly with the number of CD8+ T cells, but not with CD4+ T cells[72,74]. An independent study examining patients homozygous for the C282Y mutation showed that the low percentages of CD8+ T cells seen in the peripheral blood of HH patients were associated with low numbers of the same cells in the liver and with higher levels of hepatic tissue iron[75]. HH patients had been shown earlier to have reduced percentages of CD8+ CD28+ T cells in peripheral blood[76]. No anomalies of CD28 expression were found in the CD4 + subset. The apparent failure of the CD8+ CD28+ T cell population to expand coincided with an expansion of CD8+ CD28- T cells in peripheral blood of HLA-A3+ but not HLA-A3- HH patients[76]. Although the described abnormalities in lymphocyte populations were systematically found in the sub-population of CD8+ T lymphocytes, the association with total body iron stores is also reflected in the total lymphocyte counts. Low total lymphocyte counts were found associated significantly with a higher degree of iron overload in *HFE*-linked HH, but not in African iron overload[77]. More recently, Barton *et al* also described a significant inverse relationship of total blood lymphocyte counts and severity of iron overload in hemochromatosis probands with *HFE* C282Y homozygosity[78]. Fabio *et al* confirmed that the presence of low numbers of total lymphocyte counts and CD8+CD28+ T cells in C282Y homozygous patients was inversely related to the transferrin saturation[79]. In addition, they found low numbers of CD4+ T and NK cells, and a major increase in IL-4 and IL-10 production in the CD3+ CD8+ T cell subset[79]. A study of the Va/b T cell receptor (TcR) repertoire in a population of C282Y homozygous HH patients showed that the frequency of Va/b TcR expansions within the CD8+ pool in the group of HH patients was significantly higher in those with iron overload related pathology (9/16) than in patients (1/16) without pathology[80]. In the same study it was found that control subjects heterozygous for the C282Y mutation had an absence of expansions of the Vb5.2 and Vb12 chains in the CD8+ pool, suggesting that *HFE* could have an effect in the shaping of T cell receptor repertoire.

Functional abnormalities in CD8+ T lymphocytes were also described in HH patients. The level of autophosphorylation of the CD8-associated p56lck as well as its phosphotransferase activity, as determined by phosphorylation of an exogenous substrate, was significantly reduced by two- to three-fold in HH patients relative to a control population of healthy donors[81]. By contrast, the level of CD4-p56lck activity did not show an overall decrease relative to controls. The decreased CD8-p56lck activity seen in patients was not corrected by iron depletion. A significantly higher percentage of HLA-DR+, but not CD45RO+ cells was also found within the peripheral CD8+ T cell subset in HH patients relative to controls[76]. Moreover, functional studies showed that CD8+ cytotoxic T lymphocytes (CTL) from HH patients exhibited a diminished cytotoxic activity when compared with CD8+ CTL from healthy controls[76].

The finding of a significant association of abnormally low CD8+ T lymphocytes with a more severe clinical expression of hemochromatosis in HH patients[72,82] raises the obvious question: are if these anomalies the follow or precede the development of iron overload. The fact that those abnormalities are remarkably stable in each individual patient, that they are not corrected by phlebotomy treatment, and that they are observed in asymptomatic patients at young ages, favors the hypothesis that they are intrinsic to the genetic defect and not a consequence of the progressive iron overload. More recently it was shown that the numbers of CD8+ T lymphocytes are genetically determined, in association with other genetic determinants at the MHC class Ⅰ region close to HLA and *HFE*[83,84]. It is, therefore, conceivable that the inherited abnormalities in CD8+ T lymphocytes in HH are modifiers of the clinical expression of the disease as proposed by Cruz *et al* [82] or genetically located close to a yet unidentified modifier gene of iron metabolism.

THE REVERSE TOPIC: ADAPTIVE IMMUNITY AND IRON OVERLOAD

Genetically manipulated animal models have, therefore, become wonderful and decisive tools to address the questions of the effect on iron overload of a specific gene or protein at the systemic level. Several animal models of spontaneous iron overload were described that illustrate the influence of proteins of the adaptive immunological system on iron homeostasis, all pointing to the putative importance of the MHC class Ⅰ region.

The b2-microglobulin deficient (b2*m*$^{-/-}$) mice constitute the first described model of spontaneous iron overload[38,85,86]. These mice develop a hepatic iron overload with a distribution similar to that seen in HH liver pathology, i.e., mainly in the liver parenchyma with no evidence of iron loading in the Kupffer cells[38,86]. These mice present severe decreased cell surface expression of the MHC-class I molecules and consequently almost no CD8+ T lymphocytes[87,88]. Intestinal uptake of ferric iron and the subsequent transfer into the plasma is inappropriately increased in b*2m*$^{-/-}$ mice[86]. Upon treatment with hematopoietic cells derived from normal mice fetal liver iron overload is attenuated and shifted from the parenchymal to the Kupffer cells[86,89]. However, TfSat and intestinal iron absorption remain high, suggesting that the primary defect of iron overload is not corrected. With the discovery of the *HFE* gene and the demonstration that the C282Y mutated form failed to bind to b2m[35] it was assumed that the earlier findings in b*2m*$^{-/-}$ were due to an impaired *HFE* function. However, several subsequent studies showed that this was not sufficient to explain the pathology of b2m deficient mice. In contrast to *Hfe*$^{-/-}$, the b*2m*$^{-/-}$ mice display increased expression of the duodenal iron transporters DMT1 and ferroportin1, implicating a broader role of b2m in mammalian iron overload[90]. More recently, Rodrigues *et al* described results of a comparative study of these two models in older mice[73]. The results confirmed that the b*2m*$^{-/-}$ old mice present a more severe hepatic iron overload than the *Hfe*$^{-/-}$ counterparts. b*2m*$^{-/-}$ old mice which also showed liver steatosis, probably as a reflection of the higher hepatic iron content causing lipid peroxidation. Earlier Levy *et al* had reported the finding that in mice lacking both the *Hfe* and the b2m molecules, liver iron deposition is observed in greater levels than in mice lacking *Hfe* alone[91].

The b*2m*$^{-/-}$*-Rag1*$^{-/-}$ double knock out mice lack mature T and B lymphocytes as well as MHC-class I and Hfe expression. Rag1 is required for normal T and B lymphocyte development. b2m is required for correct folding and cell surface expression of MHC-class I like proteins. These mice present a more severe body iron overload than each of the single knock out models[39]. Besides liver iron deposition in the parenchymal cells, the b*2m*$^{-/-}$*Rag1*$^{-/-}$ also showed iron deposition in pancreas and heart. Older mice under an iron-enriched diet develop heart fibrosis, which could be prevented by treatment with normal fetal liver hematopoietic cells. To determine whether the effect of the b2m deficiency in the b*2m*$^{-/-}$*Rag1*$^{-/-}$ double knock out mouse was only due to lack of Hfe expression, double knock out mice for *Hfe* and *Rag1* were generated[92]. *Hfe*$^{-/-}$*Rag1*$^{-/-}$ double knock out mice showed increased liver iron overload compared to each of the single knock out, or the b*2m*$^{-/-}$*Rag1*$^{-/-}$. The distribution of the iron loading in *Hfe*$^{-/-}$*Rag1*$^{-/-}$ mice did not recapitulate the iron loading of the b*2m*$^{-/-}$*Rag1*$^{-/-}$, since they did not present heart or pancreas iron loading.

The d TCR$^{-/-}$ mice lack the gd intraepithelial lymphocytes. Following the administration of an iron supplemented diet, these mice showed an increased liver iron accumulation in relation to control mice[93]. In addition, d TCR$^{-/-}$ mice had a marked reduction of tumor necrosis factor alpha (TNF-a) production by intraepithelial lymphocytes when compared with controls suggesting a role for this cytokine in intestinal iron regulation.

Finally, mice deficient in the MHC class I molecules H2K^{b} and D^{b} have a strong reduction in CD8+ T-lymphocyte numbers[94]. These mice present a spontaneous increase of iron content in the liver preferentially in hepatocytes with occasionally Kupffer cells iron staining[41]. The liver iron content in this model was shown to correlate directly with the number of residual CD8+ T lymphocytes (Cardoso E and M de Sousa, unpublished observations).

The above described double knockout mouse models provide a good illustration of the modifier effect of the components of the adaptive immune system, namely a MHC class I dependent effect, on the iron overload phenotype, a conclusion also reached separately by Muckhentaler *et al* in a study of differential gene expression in b2m deficient mice[90]. Altogether the results described in animal models of hemochromatosis may help to explain why immunological anomalies modify the severity of iron overload in Hereditary Hemochromatosis[82,95].

CLOSING REMARKS

The growing knowledge and availability of genetic techniques dissecting the fine components of the host response to the challenge of infection have strengthened the opportunity of revising the topic “iron and immunity”. When the main concern for this topic resided in the effects of iron deficiency on the immune response, Weinberg pioneered the opposite concern for iron overload and infection[12]. Today it is evident that in response to infection numerous innate immunity components display metal chelating properties, including synthesis and release of lactoferrin, lipocalin and hepcidin, as discussed above. The cells involved in that response are neutrophils and macrophages. But, during evolution the macrophage, particularly the splenic macrophage, assumed the key physiological role of recognizing senescent red blood cells and recycling the iron in hemoglobin[96]. Exhaustion of that pool *in vivo* leads to “phagocytic” iron overload, such as seen in transfusional iron overload, with the expected consequences in the development of infection. If on the other hand the macrophage fails, it is to be expected that “parenchymal” iron overload will develop. This is the case in *HFE*-Hereditary Hemochromatosis. Therefore, iron overload must not be seen as one entity, but two separate entities with different relationships to immunity (**Figure 1**). Evidence was provided in this review that innate immunity is affected and affects “phagocytic” iron overload. On the reverse side, defective immune system models, namely natural and experimental defects in MHC-class I related genes (*HFE*, b2-microglobulin and HLA) are associated with the spontaneous development of “non-phagocytic” or parenchymal iron overload.

The large new question looming in the horizon of this topic with which we wish to close this brief review is: does the MHC class Ⅰ region harbor, in addition to *HFE*, other gene or genes that besides regulating lymphocyte numbers also influence the development of “non-phagocytic” or parenchymal iron overload? The search for the answer to this question will probably guide many research interests for years to come.

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