Macrophages and Systemic Iron Homeostasis

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Key Words

Hepcidin • Ferroportin • Erythrophagocytosis

Abstract

As a principal aspect of their scavenging function, splenic and hepatic macrophages phagocytize and degrade senescent and damaged erythrocytes to recover iron, mainly for the production of hemoglobin in new erythrocytes but also for other carriers and enzymes requiring iron. Splenic red pulp macrophages are specialized for iron recycling with increased expression of proteins for the uptake of hemoglobin, breakdown of heme and the export of iron. In humans, recycling macrophages contribute the majority of the iron flux into extracellular fluid, exceeding the contribution of dietary iron absorption and release of stored iron from hepatocytes. Iron release from macrophages is closely regulated by the interaction of hepcidin, a peptide hormone produced by hepatocytes, with the macrophage iron exporter ferroportin. In addition to their homeostatic role, macrophages employ multiple mechanisms to contain microbial infections by depriving microbes of iron. This review discusses the iron-scavenging function of macrophages in the context of iron homeostasis and host defense.

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Introduction

Macrophages play a key role in a vast range of biological activities, including embryonic development, scavenging and recycling, tissue repair and host defense [1]. In iron homeostasis, the macrophage is center stage as the cell that recycles senescent erythrocytes and other used-up cells into iron to be used in hemoglobin synthesis for new erythrocytes and for the synthesis of other ferroproteins. In response to systemic iron requirements, the release of iron from macrophages into plasma is regulated by the interaction of the hepatic hormone hepcidin with its receptor/iron exporter ferroportin. In humans, macrophages contribute most of the iron entering the plasma compartment. During infection and inflammation, IL-6 and other cytokines increase hepcidin synthesis, causing iron sequestration in macrophages. The resulting decrease in iron availability in tissues can limit the growth and pathogenicity of invading extracellular microbes and is an important means of host defense. Divalent metal transporters (DMTs) are used in iron-recycling macrophages to transport iron across phagosomal membranes for eventual export and are also employed by interferon- γ -activated macrophages to deplete phagosomes of iron and other divalent metals. The involve-

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ment of macrophages in iron metabolism thus serves both trophic and host defense functions. This review addresses the role of macrophages in iron metabolism in both contexts.

Iron Homeostasis

The average human male adult contains about 4 g of iron, of which about 2.5 g is in hemoglobin of red cells. In nature, dietary iron availability is usually very low, so that humans and most other vertebrates have evolved to strictly conserve and internally recycle iron. In men and nonmenstruating women, only about 1–2 mg of iron is needed to replace daily losses from desquamation and minor bleeding. By far the largest internal flux of iron serves the hemoglobin cycle, wherein iron is recovered by macrophages from the hemoglobin of old erythrocytes and supplied to erythrocyte precursors in the bone marrow for hemoglobin synthesis. Since the lifespan of erythrocytes is about 120 days, each day 20–25 mg of iron flows through the hemoglobin cycle, and 20–25 ml (or 2×10^{11}) of packed erythrocytes are removed and replaced by new ones. Because of their much lower iron content and often longer lifespan, the recycling of iron from other senescent or necrotic cells contributes much less to the iron flux. In plasma, iron is bound to transferrin, which is typically saturated to about 1/3 of its iron-carrying capacity, containing about 2 mg of iron. Total extracellular fluid volume is about 5 times larger, containing about 10 mg of iron. This means that the transferrin iron pool turns over several times a day. In the adult male, about 1 g of iron is stored predominantly in the hepatocytes and macrophages of the liver and to a lesser extent in splenic macrophages; women of reproductive age store less iron and are more prone to iron deficiency.

Systemic Iron Regulation by Hepcidin and Ferroportin

The absorption of dietary iron, its release from stores and the plasma iron concentration are subject to regulation by the iron hormone hepcidin, a 25-amino acid peptide which controls the efflux of iron from cells to extracellular fluid. The membrane protein ferroportin is the sole known conduit through which iron can transit the cell membrane [2] and it is also the receptor for the hepatic peptide hormone hepcidin [3]. Export of

iron through ferroportin is therefore a key control point for the regulation of the iron concentration in plasma. Hepcidin causes the endocytosis and eventual proteolysis of ferroportin and thereby controls iron export from macrophages and other professional iron-transporting cells, including hepatocytes, duodenal enterocytes and placental syncytiotrophoblast. The production of hepcidin in hepatocytes is feedback regulated by plasma and tissue iron concentrations as well as by the erythroid demand for iron. The bone morphogenetic protein (BMP) receptor, with its canonical transduction pathway, is the principal transcriptional regulator of hepcidin [4]. It appears that several iron sensors and adaptors modulate the sensitivity of the receptor to its iron-related ligand, BMP-6, and also affect the production of BMP-6 [5]. Although hepcidin mRNA is also detected in small amounts in macrophages, the fate and biological role of macrophage-derived hepcidin is not understood.

Iron and Host Defense

Iron is an essential trace element not only for humans and other vertebrates but also for almost every other living organism. The tenuous supply of bioavailable iron is the Achilles heel of many pathogenic microbes, and it is not surprising that defense mechanisms evolved to target this susceptibility. During infection, multiple host mechanisms act to decrease the availability of iron to the invading microbes. Upon contact with mucous epithelia, microbes encounter secretions that contain lactoferrin, an avid iron binder related to the carrier protein transferrin. When neutrophils are recruited to the site of infection, they also carry and secrete iron-sequestering lactoferrin from their secondary granules so that iron becomes unavailable to microbes that lack countermeasures for freeing it up. Both neutrophils and epithelia synthesize siderocalin (lipocalin-2, neutrophil gelatinase-associated lipocalin), a protein which avidly binds certain siderophores, small organic iron chelators used by microbes to secure iron from the environment. Within hours after infection, the concentration of iron bound to transferrin is greatly decreased (hypoferremia) by inflammatory signals that override the normal homeostatic regulation of iron concentrations and fluxes.

Fig. 1. Flow of iron in macrophages. Macrophages take up iron through erythrophagocytosis of damaged or senescent erythrocytes. Macrophages can also scavenge heme and hemoglobin, usually complexed with hemopexin and haptoglobin, respectively, and endocytosed by CD163 and CD91, respectively. Whether phagocytosed in erythrocytes or endocytosed by scavenging, hemoglobin undergoes proteolysis to release heme. Heme is degraded by HO-1 to release iron, which is exported to the cytoplasm by DMT1 and probably also by Nramp1. Cytoplasmic chaperones of the poly(rC) binding protein (PCBP) family deliver iron for storage in ferritin. Alternatively, iron from endosomes or phagolysosomes may also be delivered by an unknown carrier to ferroportin (Fpn) for export. When demand for iron is high, ferritin is degraded by lysosomes or proteasomes, and the released ferrous iron is exported through ferroportin. The rate of iron export is controlled by extracellular hepcidin through its ability to induce the endocytosis and proteolysis of ferroportin. The ferroxidase ceruloplasmin oxidizes iron to its ferric form and thereby facilitates iron export and loading onto the carrier protein transferrin.

Altered Iron Homeostasis during Infection and Inflammation

The induction of inflammatory cytokines results in an acute-phase response, the reprogramming of the synthetic repertoire of the liver to serve host defense. IL-6, and to a lesser extent IL-1, act to increase the transcription of the iron-regulatory hormone hepcidin [6] and increase the synthesis of a number of proteins involved in the scavenging and sequestration of iron in tissues, including ceruloplasmin, siderocalin, haptoglobin, hemopexin and ferritin. Increased hepcidin binds to macrophage ferroportin and causes its endocytosis and degradation, thereby trapping iron in cytoplasmic ferritin of macrophages that recycle aged erythrocytes and causing the characteristic hypoferremia and eventually anemia of inflammation. On the other hand, transferrin synthesis is decreased, which acts to preserve the supply of diferric transferrin for erythropoiesis in the face of inflammation-induced hypoferremia. Iron sequestration and hypoferremia may be protective, as illustrated by the increased susceptibility of patients with hepcidin defi-

ciency and iron overload to infections with highly iron-dependent microbes, such as *Yersinia* and *Vibrio* species [7–9].

Iron and Macrophages

The macrophage is a key agent in iron homeostasis as well as in inflammatory hypoferremia. Unlike other cell types which take up iron predominantly in the form of diferric transferrin, macrophages in the spleen, in the liver (Kupffer cells) and perhaps elsewhere recognize damaged or senescent erythrocytes, phagocytize them and digest them to extract heme and eventually iron [10] (fig. 1). In laboratory rats, it has been estimated that each of these macrophages ingests about one erythrocyte per day, without any apparent harmful effect on the macrophage, but higher rates of erythrophagocytosis, as may occur during hemolytic diseases, may cause injury to macrophages [11]. Heme is degraded through the action of heme oxygenase (HO; predominantly HO-1) to release iron into the cytoplasm for eventual export by plasma

membrane ferroportin (fig. 1). In humans, iron flux from macrophages greatly exceeds inflow from dietary iron absorption and from iron stored in hepatocytes. In laboratory mice, which consume much more food relative to their body mass, the iron flow from enterocytes is comparable to that from macrophages.

Erythrophagocytosis

Erythrocytes undergo many changes as they age, but in humans they are not normally ingested by macrophages until they are close to their terminal age of about 120 days or undergo eryptosis [12, 13], a suicidal process distantly resembling apoptosis but occurring in the absence of nuclei or mitochondria and triggered by chemical or osmotic stress or energy depletion. Based on the fraction of blood flow to the spleen, each human erythrocyte transits the splenic circulation every 20 min, where it is subjected to several types of quality control [14]. Erythrocytes come into contact with macrophage receptors in the red pulp probing for the display of opsonic antibodies or pathological surface markers and are subjected to deformability challenge as erythrocytes return to the circulation through narrow endothelial slits. Aging changes must reach a specific threshold that is recognized by macrophages, thus initiating erythrophagocytosis. Alterations that may be recognized by macrophages as markers of erythrocyte aging [15–18] include the following: (1) modifications of the most abundant erythrocyte membrane protein, Band 3, inducing its membrane clustering (probably the most important factor); (2) the appearance of phosphatidylserine on the outer leaflet of the plasma membrane, where it can be recognized by specific macrophage or endothelial receptors, or opsonins that bind to phosphatidylserine [17, 19]; (3) increased membrane rigidity, and possibly (4) the loss of sialic acid and the CD47 antigen. Clustering of Band 3 protein is caused by the binding of denatured hemoglobin to its cytoplasmic domain and may be promoted also by age-related proteolytic cleavage or covalent modifications. The modified and clustered Band 3 protein is recognized by opsonic natural antibodies and complement, triggering conventional antibody- and complement-mediated phagocytosis. Eryptosis [13] is initiated in many pathological states that injure erythrocytes, including systemic infections, malaria, hemoglobinopathies and genetic defects of erythrocyte metabolism. Eryptotic cells are shrunken, with blebbed membranes and phosphatidylserine exposed in the outer leaflet of the plasma mem-

brane. Phosphatidylserine receptors and phosphatidylserine-specific opsonins are particularly important for the phagocytosis of eryptotic erythrocytes. The erythrophagocytosis of stressed or damaged erythrocytes by macrophages also has a host defense function because it removes from circulation cells that have been parasitized by malarial or other parasites of erythrocytes. The partial protection of subjects with mild genetic erythrocyte traits (sickle cell trait, glucose-6-phosphate dehydrogenase deficiency and others) from lethal malaria could be explained in part by the enhanced clearance of parasitized erythrocytes in these conditions [20].

Recovery of Iron from Erythrocytes

In the phagocytic vacuole, the erythrocyte is exposed to reactive oxygen species and hydrolytic enzymes, leading to the release of hemoglobin and eventually heme into vacuolar fluid (fig. 1). There the heme-inducible enzyme HO-1 uses molecular oxygen and NADPH-reducing equivalents to cleave heme into equimolar amounts of iron, carbon monoxide and biliverdin [21, 22]. Biliverdin is then reduced to bilirubin by biliverdin reductase. HO-1 is a membrane-anchored protein highly expressed in the liver and spleen and primarily but not exclusively located in the endoplasmic reticulum, from where it can be delivered when this compartment contributes to the formation of the phagosomal membrane. HO-1 is essential for the recovery of iron from heme, as indicated by the consequences of HO-1 deficiency on iron homeostasis in mouse models [21, 22]. Ablation of HO-1 results in an iron-restricted anemia with low serum iron, low transferrin saturation and low erythrocyte mean corpuscular volume (MCV) but iron deposition in the liver and other tissues. This pattern is indicative of a defect in iron recycling accompanied by maldistribution of stored iron. The many other manifestations of HO-1 deficiency in mice and two human patients [23, 24] are presumed to result from the toxicity of free heme normally neutralized by HO-1, or the role of HO-1 and its enzymatic products in the regulation of inflammation and apoptosis. In HO-1-deficient patients and mice, macrophages are among the cell types primarily affected by heme toxicity, and the destruction of erythrophagocytosing macrophages in the spleen and liver then further exacerbates heme-mediated injury to endothelia and other tissues [22]. A second HO, HO-2, is constitutively expressed in many tissues but at a lower level, apparently insufficient to compensate for HO-1 deficiency.

Alternative Recycling Pathways

Although most senescent or damaged erythrocytes are removed from the circulation by macrophages, intravascular hemolysis may reach comparable levels under pathological or stress conditions, including such common situations as vigorous exercise. Ruptured erythrocytes release hemoglobin into plasma, where hemoglobin binds to its dedicated carrier, haptoglobin. Hemoglobin-haptoglobin complexes are taken up by hepatocytes and macrophages via the CD163 receptor [25], and the iron is recovered by processes similar to those that follow erythrophagocytosis (fig. 1). Oxidation of hemoglobin that results in the oxidation of its heme-associated ferrous iron to ferric iron causes the release of the resulting heme (i.e. heme containing ferric instead of ferrous iron) into the circulation, where it binds to hemopexin. Hemopexin-heme complexes are cleared by hepatocytes and macrophages via the CD91 receptor [26], and the iron is extracted by HO. In HO-1-deficient mice and humans, the loss of macrophages engaged in iron recycling [22] causes intravascular hemolysis and the release of hemoglobin, leading to the generation of hemoglobin-haptoglobin complexes. At the same time, increased release of heme into the circulation generates heme-hemopexin complexes. The complexes are taken up by hepatocytes and other tissues expressing the CD163 and CD91 receptors [27], where they cause abnormal iron deposition, presumably facilitated by remaining constitutive HO-2 activity.

Specialization of Macrophages for Erythrophagocytosis

Splenic red pulp macrophages are a specialized highly erythrophagocytic cell type, characterized in mice as F4/80^{hi}CD68⁺CD11b^{lo/-} splenic cells with intense autofluorescence [28]. Their development is dependent on the expression of the transcription factor Spi-C. Spi-C is selectively expressed in red pulp macrophages. Mice null for Spi-C lack this macrophage subset but not other macrophage subtypes in the spleen or other tissues. Spi-C-deficient mice accumulate iron in the spleen consistent with their low expression of splenic macrophage ferroportin, resulting in a restricted capacity for exporting recycled iron. Spi-C is induced by M-CSF but to a much lesser extent by GM-CSF, consistent with the proposed role of these growth factors in the differentiation of macrophages for erythrophagocytosis and other scavenging tasks versus inflammation/host defense. Compared to

other macrophage subtypes, red pulp macrophages show increased expression of genes required for erythrophagocytosis and iron recycling (CD163, ferroportin, HO) and the adhesion molecule VCAM-1. VCAM-1 appears to be directly under Spi-C control, but the other molecules characteristic of erythrophagocytosis may be induced by exposure of macrophages to heme and iron rather than directly by Spi-C [29]. Other factors that influence the development of the erythrophagocytic phenotype include inflammatory cytokines. Murine and human macrophages treated with interferon- γ (M1 polarization) express lower levels of ferroportin and HO-1 than macrophages treated with IL-4/IL-13 (M2 phenotype), and the latter have a greater capacity for iron export [30, 31]. In concert with their scavenging and tissue repair phenotype, M2 macrophages also take up heme-hemopexin and hemoglobin-haptoglobin complexes, using CD163 and CD91 receptors, respectively, both of which are highly expressed under M2 conditions (fig. 1). Altogether, more than 60% of iron-related genes are differentially expressed in M1 versus M2 macrophages [32]. M2 macrophages supported faster growth of tumor cells, and this effect was reversed by the chelator desferoxamine, implicating iron as a tumor growth stimulant released by tumor-associated macrophages, which are M2-like [32]. The effect of macrophage polarization on iron release or retention is cell-autonomous, and the block to iron export in M1 macrophages would be further amplified in vivo by the effect of hepcidin, expected to be present at higher concentrations in classically inflamed environments.

Regulation of Macrophage Phenotype by Iron and Heme

In addition to factors present in the macrophage environment, iron and heme release in the macrophage induce the expression of molecules necessary for iron recycling. HO-1 transcription is increased by the heme-dependent dissociation of the repressor Bach1 from multiple sites in the HO-1 promoter (Maf recognition elements), allowing the Maf activators to bind to these sites to activate HO-1 transcription [33]. Both activators and repressors heterodimerize with Nrf transcription factors. Ferroportin and ferritin transcription is also induced by heme by similar mechanisms [29, 34, 35]. In addition, iron increases the synthesis of ferritin and ferroportin by relieving the translational block mediated by the attachment of iron-regulatory proteins 1 and 2 to the 5' iron-regulatory elements in their mRNAs [36].

Subcellular Trafficking of Recovered Iron in Macrophages

The transport of recycled iron within the macrophage requires the movement of iron across the phagosomal membrane, chaperoned transport through the cytoplasm and delivery of ferrous iron to plasma membrane ferroportin, the nonredundant iron exporter (fig. 1). In addition, ferric reductases and ferroxidases participate in transport by facilitating the interconversion of ferrous and ferric iron to match the chemical requirements of iron transporters and storage mechanisms. Cytoplasmic ferritin serves to store macrophage iron when systemic demand for iron is low or inflammation leads to iron sequestration in macrophages. Two related ferrous iron transporters, DMT1 [also called natural resistance-associated macrophage protein (Nramp) 2] and Nramp1, are required for normal export of iron from phagosomes [37] and appear to function in a partially redundant manner. The subsequent journey of iron through the cytoplasm must be facilitated by chaperones that negate the chemical reactivity and toxicity of iron, but the identity of these molecules is unknown. A family of chaperones, human poly(rC) binding proteins 1–4, has been implicated in the delivery of iron to ferritin [38] and to certain other non-heme ferroproteins, but it is not clear if the same or different proteins deliver cytoplasmic iron for iron export from macrophages.

Iron Export from Macrophages

Although the details of iron transport through ferroportin are not yet understood, it transports ferrous iron and requires a ferroxidase to convert the iron to the ferric form and to load it onto its plasma carrier transferrin. The copper-containing ferroxidase ceruloplasmin catalyzes the oxidation reaction and thereby facilitates iron efflux [39] (fig. 1), as indicated by the impaired export of iron from macrophages and hepatocytes in ceruloplasmin-deficient mice and humans. In addition to the abundant soluble ceruloplasmin originating from hepatocytes and circulating in plasma, a membrane-anchored glycosylphosphatidylinositol-containing form of ceruloplasmin predominates in all other tissues, including the brain. Both forms may stabilize ferroportin by facilitating the flow of ferrous iron through the molecule. Plasma transferrin is the ultimate carrier of ferric iron, with most of the cargo destined for erythropoiesis, which is wholly dependent on transferrin-bound iron. When the carry-

ing capacity of transferrin is exceeded in iron overload or in transferrin deficiency, nontransferrin-bound iron is generated [40], consisting of iron associated with albumin or with small organic molecules such as citrate or acetate. This form of iron is avidly taken up by hepatocytes but also by cardiac myocytes, pancreas and endocrine tissues not normally involved in iron storage and accounts for the development of liver disease and other organ damage in hereditary hemochromatosis and other iron overload diseases.

Iron Transporters in Macrophages and Their Subcellular Distribution

Macrophages express iron transporters that are capable of moving iron across the membrane in either direction, i.e. DMT1 into the cytoplasm and ferroportin out of the cytoplasm. In other cell types, e.g. the duodenal enterocyte, the importer DMT1 is on the apical membrane and the exporter ferroportin on the basolateral membrane, effecting the flow of iron from the intestinal lumen to the blood plasma. In macrophages, there must be equivalent segregation of the importers and exporters, presumably to the phagosomal membrane and the plasma membrane, respectively. However, the phagosomal membrane originates in the plasma membrane, so the process requires either subcellular storage of the importers and exporters with selective delivery to different membranes or some other mechanism for their segregation. Cytoplasmic vesicles containing ferroportin have been observed in resting macrophages [41] and apparently translocate to the cell membrane when macrophages are iron-loaded or subjected to erythrophagocytosis. It remains to be seen how iron importers traffic to their sites of activity and how the trafficking of iron transporters in macrophages is regulated in response to iron loading or other stimuli.

Macrophage Iron and Host Defense

As its name suggests, the iron transporter Nramp1 (SLC11a1 in systematic nomenclature) expressed in murine macrophages mediates their resistance to intracellular microbes, including *Mycobacterium bovis* BCG, *Leishmania donovani* and *Salmonella typhimurium* [42]. Mice or humans deficient in Nramp1 have increased susceptibility to infections with these or closely related pathogens. In agreement with its role in host defense,

Nramp1 synthesis is induced in macrophages activated by interferon- γ [43]. In further support of its proposed role, Nramp1 is not only found in macrophages but is also abundant in human neutrophils in the membranes of gelatinase-containing granules and is delivered to phagosomes during phagocytosis. There is controversy regarding whether Nramp1 functions similarly to DMT1 (Nramp2) to move iron and other divalent metals along a proton gradient, i.e. out of phagosomes and into the cytoplasm [44], or in the opposite direction, into the phagosome [45], to elicit antimicrobial activity via the Fenton-Haber-Weiss reactions that generate the toxic hydroxyl radical.

The Effect of Iron on the Immune and Inflammatory Function of Macrophages

Like erythrophagocytic macrophages in the spleen and liver, macrophages in hemorrhagic tissues (e.g. physical injury, hemorrhagic stroke, bleeding into an atheroma) must also scavenge damaged or lysed erythrocytes. A recent study [46] indicated that iron-loaded macrophages in chronic venous leg ulcers exhibit a phenotype that promotes inflammation by increased release of TNF- α and reactive oxygen species and retards wound healing by causing DNA damage to fibroblasts and activating their senescence program. In experimental models of skin injury in mice, these effects could be reproduced by coadministering iron-dextran and ameliorated by desferrioxamine, suggesting that they were iron-dependent. However, proteomic analysis of human monocyte-macrophages cultured in vitro with hemoglobin-haptoglobin complex-

es [47] showed the expected increase in proteins involved in hemoglobin and heme clearance and in antioxidant proteins, with concomitant suppression of HLA class II proteins, indicating that the in vivo observations of the iron-dependent proinflammatory macrophage phenotype are not simply a result of exposure to extravascular hemoglobin-haptoglobin complexes but most likely require an as yet unidentified cofactor. The benign resolution of most simple hematomas favors this interpretation.

Conclusion and Future Directions

Iron scavenging, recycling and storage by macrophages serves important trophic and host defense functions. Although much has been learned about the mechanisms involved, further work is needed to provide a detailed understanding of iron movement in the macrophage and the subcellular location and regulation of molecules that mediate it. The contribution of extracellular or phagosomal iron deprivation to host defense against specific pathogens is also of great interest and, if understood, could be manipulated for therapeutic purposes. Finally, the effect of macrophage iron and other erythrocyte breakdown products on inflammatory processes in various pathological settings should be a fruitful area for future studies.

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