9

Impact of Metals on Immune Response and Tolerance, and Modulation of Metal Metabolism during Infection

Günter Weiss

Abstract

Several metals play important roles in host cell and microbial metabolism because they form a part of central enzymes that are essential, for example, for DNA synthesis, cellular respiration, and key metabolic pathways. The availability of these metals differentially impact on host antimicrobial immune responses as well as on microbial defenses against them (Botella et al. 2012). Thus, during infection, host cells attempt to gain sufficient access to these metals or to limit the availability of these factors for microbes; this is thought to play a decisive role in the course of infections. Accordingly, subtle changes in the metabolism of these metals and their distribution throughout the body occur: microbes activate different pathways to secure a sufficient supply of these metals needed for their pathogenicity and proliferation as well as to mount effective defenses against the host immune system.

This review focuses on the role of iron in the host–pathogen interplay. A brief discussion is included on the role of zinc, manganese, and copper for host–pathogen interaction, immune function, and their alteration by the inflammatory response.

Introduction

Metal ions are pivotal in host–pathogen interactions. This is partly because microbes need metals, such as iron (Fe), zinc (Zn), copper (Cu), or manganese (Mn), for important metabolic processes and proliferation and as central components for defenses against host-mediated radical formation. The basis for the essential function of these metals can be traced back to their ability to accept or donate electrons needed during metabolic processes. Metal accumulation

can become toxic, however, due to their ability to catalyze the formation of toxic oxygen and nitrogen radicals, which can intoxicate microbes or damage surrounding cells and tissues. Microbes take up transition metals through multiple pathways, and sufficient acquisition of these metals through pathogens is linked to the pathogenicity and proliferation of microbes. In additional, transition metals (specifically iron) play important roles in antimicrobial host responses, not only by synergistic effects toward antimicrobial radical formation but also by directly affecting immune cell proliferation and antimicrobial immune effector pathways. Thus, the host immune system affects the metabolism of these metals and/or their availability for microbes via the action of cytokines, cellular proteins, or hormones—for which the term *nutritional immunity* has been coined—and plays a decisive role in the course of infection.

Role of Iron for Immune Cell Plasticity

Iron is an essential metal due to its role as a prosthetic group in several essential proteins and enzymes involved in metabolic processes, mitochondrial respiration, and DNA synthesis. Iron catalyzes the formation of hydroxyl radicals, which then modulate the binding affinity of critical transcription factors, such as HIF-1 or NF-kB, thus affecting gene expression during inflammation (Rosen et al. 1995). Therefore, both Fe overload and Fe deficiency exert subtle effects on essential metabolic pathways as well as on the growth, proliferation, and differentiation of cells. Accordingly, the availability of iron affects the proliferation and differentiation of immune cells, which exploit different pathways to acquire this metal.

The close interaction between iron and immunity is underscored by observations that certain immunological proteins do alter cellular Fe metabolism, as described for β2 microglobulin, HFE (which is a nonclassical MHC-I molecule linked to the majority of cases with human hemochromatosis), tumor necrosis factor receptor (TNF-R), and the natural resistance-associated macrophage protein (NRAMP1). Changes in immune function thus affect Fe homeostasis and vice versa (Weiss 2002).

Lymphocytes have evoked different mechanisms to acquire iron, even under conditions when Fe availability is limited. All lymphocytes subsets, which include B and T lymphocytes as well as natural killer (NK) cells, are dependent on transferrin/transferrin receptor (TfR)-mediated Fe uptake; blockade of this pathway leads to diminished proliferation and differentiation of these cells (Seligman et al. 1992). Accordingly, mitogenic stimuli, such as phytohemagglutinin, increase TfR surface expression on B and T cells. However, lymphocyte subsets differ in their dependence on transferrin-mediated Fe uptake. Induction of experimental Fe overload in rats resulted in a shift in the ratio between T helper (CD4+) and T suppressor/cytotoxic T cells (CD8+), with a relative expansion of the latter. Moreover, even the T helper

(Th) subset responds differently to Fe perturbations. Several subsets of CD 4+ T helper cells are well established in humans; these are termed type 1 (Th-1), Th-2, Th-9, Th-17, and Treg. Each subset produces a typical set of cytokines that regulate different immune effector functions, which cross-react with each other and play a decisive role in host responses to infections.

Whereas Th-1 cells are very sensitive to treatment with anti-TfR antibodies, resulting in inhibition of their DNA synthesis, Th-2 cells are resistant to this procedure. This may be because Th-2 clones exhibit larger chelatable Fe storage pools than Th-1 cells. Thus, Th-1-mediated immune effector pathways are much more sensitive to changes in Fe homeostasis *in vivo* (Thorson et al. 1991). The latter can partly be attributed to a direct regulatory effect of iron on the activity of the central regulatory Th-1 cytokine IFN-γ (Nairz et al. 2013).

Monocytes and macrophages are the conductors that orchestrate Fe homeostasis in health and disease; they are thus on the interface between Fe homeostasis and immunity. One reason for this is that macrophages are essential for the maintenance of a sufficient supply of iron for erythropoiesis, which is achieved by recycling iron from senescent red blood cells taken up by macrophages via erythrophagocytosis (Weiss and Schett 2013). In addition, macrophages need iron to produce highly toxic hydroxyl radicals by the enzyme phagocyte oxidase as part of their antimicrobial armamentarium (Rosen et al. 1995). At the same time, macrophages are centrally involved in the diversion of Fe traffic under inflammatory conditions that occur during infection or cancer.

Alterations of Iron Metabolism in Inflammation

Based on the decisive role of iron for both the host immune system and microbes, it has become clear that Fe metabolism is significantly affected during the course of infection. These alterations of Fe traffic are thought to result from a defense strategy of the body to limit the availability of iron for invading pathogens, for which the term *nutritional immunity* has been coined (Nairz et al. 2010; Cassat and Skaar 2013). This alteration in Fe homeostasis is mediated by cytokines and radicals as well as by acute phase proteins, which originate primarily from the liver. Combined, this mediation leads to retention of iron in macrophages and an impaired Fe absorption from the diet (Weiss and Schett 2013).

The contribution of cytokines to systemic Fe regulation was first confirmed through the observation of sustained hypoferremia in mice injected with TNF-α or IL-1. Hypoferremia was paralleled by hyperferritinemia, which was traced back to induction of ferritin transcription by cytokines in cells of the reticuloendothelial system. In addition, the proinflammatory cytokines IL-1 and IL-6 regulate ferritin expression at the translational level. Iron storage in macrophages is further promoted via increased phagocytosis of erythrocytes.

This occurs when erythrocytes are damaged, as a result of exposure to inflammation-driven radical formation, and are phagocytized via C3bi (CD11b/CD18) receptors, which increase in expression following TNF-α treatment. Accordingly, the application of sublethal doses of TNF-α to mice resulted in a shortening of erythrocyte half-life and a faster clearance of these cells from the circulation via erythrophagocytosis (for a review, see Weiss 2002).

The intriguing relationship between immunity and Fe homeostasis took on a new dimension after the acute phase protein hepcidin was identified as the master regulator of Fe homeostasis (Ganz 2009). The observation that hepcidin-deficient mice injected with turpentine did not develop hypoferremia suggested that hepcidin could be involved in Fe disturbances during inflammation or infection. One underlying mechanism is the induction of hepcidin expression by lipopolysaccharides (LPS), IL-1, IL-6, or IL-22 in hepatocytes. Hepcidin acts on Fe homeostasis upon binding to the only known Fe export protein ferroportin, thereby leading to Fe retention in macrophages and impaired Fe absorption from the diet (Ganz 2009). Accordingly, increased circulating hepcidin concentrations in serum result in reduced circulating Fe levels and increased Fe retention in macrophages, a scenario which should limit the access to iron from circulating pathogens (Ganz 2009; Nairz et al. 2010). In addition, mammalian monocytes and macrophages produce small amounts of hepcidin in response to LPS or IL-6. Although the basal expression is relatively low in comparison to the amount of hepcidin produced in the liver, microbial challenges, such as group A *streptococci* and *Pseudomonas aeruginosa*, can induce a 20- to 80-fold increase of hepcidin expression in these cells through a TLR-4-dependent pathway, whereas IL-6-mediated induction of hepcidin is mediated via STAT3 activation (Nairz et al. 2010; Ganz 2009). Interestingly, hepcidin released by macrophages targets ferroportin in an autocrine fashion, thus promoting macrophage or monocyte Fe accumulation during inflammatory processes. This may be a fast-acting defense mechanism of the innate immune system against invading microbes. Hepcidin targets ferroportin exposed on the cell surface, resulting in immediate blockage of Fe release and reducing the availability of this essential microbial nutrient in circulation.

Effects of hepcidin on Fe homeostasis appear, however, to occur rapidly, but only for a limited duration. This has been confirmed by the observation that injection of LPS results in the induction of hepcidin and development of hypoferremia, which lasted for several hours (Kemna et al. 2005). Thereafter, serum Fe concentrations returned to normal or were even higher than at baseline. Thus, to ensure a sustained modulation of Fe homeostasis under inflammatory conditions and during infection, a concerted action of different signals exerted by cytokines, acute phase proteins, and hormones is mandatory. A central regulatory factor that ensures sustained Fe retention in monocytes or macrophages, as well as reduced expression of ferroportin during inflammation, is IFN-γ. The Th-1-derived cytokine IFN-γ induces

ferritin transcription but also affects ferritin translation, which is based on activation of iron regulatory protein (IRP)-binding affinity by the cytokine. This is partly due to the stimulation of nitric oxide (NO) formation by IFN-γ, which then activates IRP-1 binding to the ferritin iron-responsive element (IRE). This leads to an inhibition of ferritin translation, whereas effects to IRP-2 activity depend on the type of cell and NO product (Pantopoulos et al. 2012). Moreover, hydrogen peroxide and superoxide anion modulate IRP-1 activity through a rapidly inducible process involving kinase or phosphatase signal transduction pathways; this results in posttranscriptional regulation of IRE-regulated target genes, such as TfR and ferritin (Recalcati et al. 2010; Pantopoulos et al. 2012). IFN-γ treatment of monocytes blocks the uptake of transferrin-bound iron via downregulation of TfR expression. This is most likely due to induction of a proximal inhibitory factor by IFN-γ, which inhibits TfR transcription. However, IFN-γ induces the expression of divalent metal transporter 1 (DMT1) and acts synergistically with LPS and TNF-α in this respect. This stimulates ferrous Fe uptake into these cells and promotes their incorporation into ferritin (Ludwiczek et al. 2003). At the same time, IFN-γ and LPS induce Fe retention in macrophages by downregulating ferroportin transcription, thus blocking Fe release from these cells (Ludwiczek et al. 2003). Hepcidin formation by macrophages or monocytes may be part of a fast-acting innate immune effector arm aimed at preventing Fe export from macrophages, which is relevant under microbial invasion. The outcome is to reduce circulating Fe concentrations and the availability of this nutrient for pathogens. Thereafter, ferroportin transcription is blocked by IFN-γ or LPS, thus ensuring a prolonged blockage of Fe export.

While anti-inflammatory cytokines (such as IL-4, IL-10, or IL-13) do not affect the suppression of ferroportin mRNA expression by IFN-γ or LPS (Ludwiczek et al. 2003), treatment of murine macrophages with IL-4 and/or IL-13, prior to stimulation with IFN-γ, suppresses NO formation and subsequent IRP activation, which concomitantly enhances ferritin translation. This has also been found to be true in human monocytic cells (THP-1), which do not express detectable amounts of inducible nitric oxide synthase (iNOS). Conversely, TfR mRNA levels increase following pretreatment of IFN-γ-stimulated macrophages with the anti-inflammatory cytokines. This may be referred to as IL-4- or IL-13-mediated antagonization of the inhibitory signal induced by IFN-γ, which inhibits TfR expression by an IRP-independent pathway. In addition, IL-10 and IL-6 may affect macrophage Fe acquisition by stimulating the expression of the hemoglobin scavenger receptor CD163, thus promoting the uptake of hemoglobin-haptoglobin complexes into monocytic cells. The important role of Th-2-derived cytokines for the development of hyperferritinemia under chronic inflammatory processes was confirmed by a clinical study in patients with Crohn disease. In a placebo-controlled, double-blinded study, patients who received therapy with human recombinant IL-10 developed a normocytic anemia preceded by a significant increase in serum ferritin levels;

reticulocyte counts were not, however, affected, compared to placebo-treated controls (Weiss and Schett 2013). Both anemia and hyperferritinemia resolved spontaneously within two to four weeks after IL-10 therapy was terminated. Thus, Th-2-derived cytokines may increase Fe uptake via induction of TfR and CD163, but will also promote Fe storage within ferritin through activated macrophages. In addition, IL-10 stimulates HO-1 expression and activity, thus promoting Fe reutilization from phagocytosed erythrocytes, hemoglobin-haptoglobin complexes, and hemopexin-bound heme, respectively (Gozzelino et al. 2012). In addition, the increased expression of ferritin and the subsequent sequestration of iron within this protein contribute to limiting tissue damage during infection by inhibiting the catalytic action of iron. Thus, overexpression of H-chain ferritin (which harbors ferroxidase activity) results in tolerance to infection by limiting inflammation-induced tissue damage, as shown in animal models of malaria (Gozzelino et al. 2012).

In summary, pro- and anti-inflammatory cytokines and, most importantly, acute phase proteins cooperate at multiple steps to increase macrophage Fe accumulation by stimulating various Fe acquisition pathways in these cells. At the same time, cytokines and hepcidin inhibit Fe export from macrophages by downregulating ferroportin expression. This results in Fe retention within cells of the reticuloendothelial system and Fe-restricted erythropoiesis (Table 9.1).

These processes result in the development of hypoferremia, hyperferritin emia, and Fe-restricted anemia, the latter of which has been termed anemia of chronic disease (ACD) or anemia of chronic inflammation (Weiss and Schett 2013). In a clinical setting, ACD occurs frequently in patients suffering from chronic inflammatory disorders (e.g., autoimmune diseases, chronic infections, malignancies). Although the development of anemia is associated with detrimental effects, especially in relation to cardiac function, quality of life, and growth and mental development, the underlying hypoferremia and the diversion of iron from circulation may also exert potentially positive effects, especially when infections underlie chronic immune activation.

Impact of Iron on Antimicrobial Immune Effector Function

The development of ACD does not only limit the availability of iron for microbes but can strengthen the immune response directed against invading pathogens. Specifically, Fe loading of monocytes or macrophages inhibits IFN-γ-mediated pathways, such as the formation of TNF-α, reduced expression of MHC class II antigens and ICAM-1, decreased formation of neopterin, and impaired tryptophan degradation via IFN-γ-mediated induction of indole-amine-2,3–dioxygenase (for a review, see Nairz et al. 2010). As a result, Fe-loaded macrophages have an impaired potential, *in vitro* and *in vivo*, to kill various bacteria, parasites, fungi (e.g., *Legionella*, *Listeria*, *Ehrlichia, Mycobacterium, Salmonella*, *Leishmania*, *Plasmodium*, *Candida, Mucor*), and

Table 9.1 Pathways for regulating Fe homeostasis through cytokines, acute phase proteins, and radicals: interleukin (IL), tumor necrosis factor (TNF), interferon (IFN), divalent metal transporter 1 (DMT1), ferroportin (FP1), nitric oxide (NO), and hydrogen peroxide (H_2O_2).

Factors	Mechanisms
TNF-α	Induces ferritin transcription, which promotes iron storage within cells of the reticuloendothelial system Shortage of erythrocyte half-life (TNF-α) and stimulation of erythrophagocytosis Inhibits hepcidin formation Blocks iron absorption from the duodenum
IL-1	Stimulates ferritin transcription and translation and promotes macrophage iron retention Stimulates hepcidin formation
IL-6	Induces ferritin transcription and translation Stimulates hepcidin formation Stimulates CD163 and increases the uptake of hemoglobin-haptoglobin complexes by macrophages Induces heme oxygenase and heme degradation
IFN-γ	Stimulates DMT1 synthesis and increases uptake of ferrous iron into monocytes Downregulates FP-1 expression, which inhibits iron export from macrophages Downregulates TfR via induction of a proximal inhibitory signal
IL-4, IL-10, IL-13	Increases TfR expression and transferrin-mediated iron uptake into inflammatory macrophages Stimulates ferritin translation by inactivating IRP and decreasing NO expression IL-10 stimulates CD163 and increases the uptake of hemoglobin-haptoglobin complexes by macrophages IL-10 stimulates heme oxygenase expression and heme degradation
IL-22	Stimulates hepcidin expression
NO	Stimulates IRP-1 binding affinity, thus blocking ferritin translation and stabilizing TfR mRNA (feedback regulation with iron; NO formation is affected via modulating iNOS expression) Induces ferroportin expression via Nrf2 activation Modulates IRP-2 expression and stability
Oxygen radicals	H_2O_2 when applied extracellularly stimulates IRP-1 activity with blocking of ferritin translation and stabilizing TfR mRNA Superoxide anion formed intracellularily inhibits IRP binding affinity
Hepcidin	Formed upon stimulation of mice with LPS and several proinflammatory cytokines Blocks iron egress from macrophages Inhibits duodenal iron absorption Exerts autocrine regulation of iron export from inflammatory macrophages

viruses through IFN-γ mediated pathways. This can partly be attributed to the reduced formation of NO in the presence of iron, since NO is an essential effector molecule of macrophages to fight infectious pathogens and tumor cells. Iron blocks the transcription of inducible NO synthase (iNOS or NOSII), the enzyme being responsible for cytokine-inducible high output formation of NO by hepatocytes or macrophages. In addition, by inhibiting the binding affinity of the transcription factors NF-IL6 and the hypoxia inducible factor-1 to the iNOS promoter iron, iron impairs iNOS inducibility by cytokines (Nairz et al. 2010). According to the regulatory feedback loop, NO produced by activated macrophages leads to an inhibition of ferritin translation, thus linking the maintenance of Fe homeostasis to NO formation for host defense. Through its deactivating effect on IFN-γ function, iron also affects the Th-1/Th-2 balance: Th-1 effector functions are weakened while Th-2-mediated cytokine production (e.g., IL-4 activity) is increased, a condition that is rather unfavorable in an infection (Nairz et al. 2010; Mencacci et al. 1997). Iron overload also has negative effects on neutrophil function: in chronic hemodialysis patients, Fe therapy impairs the potential of neutrophils to kill bacteria, thus reducing their capacity to phagocytize foreign particles (Weiss 2002). By modulating cytokine activities, iron triggers macrophage polarization, and opposite M1 and M2 macrophages differ in contrasting metabolic profiles in regard to Fe homeostasis (Recalcati et al. 2010). Moreover, the induction of M2 polarization along with increased expression of HO-1 has been linked to immune tolerance in infections, specifically in malaria (Gozzelino et al. 2012).

Thus, both Fe overload and Fe deficiency have unfavorable immunological effects *in vivo*. Accordingly, mice kept on an Fe-rich diet presented with a reduced production of IFN-γ, compared to mice fed with a normal diet, and animals that received an Fe-deficient diet showed decreased T cell proliferation (Omara and Blakley 1994). Both Fe-overloaded and Fe-deficient mice had an increased mortality when a sublethal dose of LPS was received, compared to animals with a normal Fe status.

In one study, Fe-deficient children had a reduced incidence of infection accompanied by a higher percentage of CD8+ cells producing IL-6, a more pronounced expression of T cell activation markers on lymphocytes, and an increased formation of IFN-γ, as compared to Malawian children with a normal Fe status (Oppenheimer 2001). This coincides with the observation of an adverse outcome in children who received Fe supplementation, mainly as a result of an increased incidence of severe malaria, bacterial infection, or diarrhea (Sazawal et al. 2006; Soofi et al. 2013). In line with this observation, improved outcomes, but no mortality benefit, were observed in children with cerebral malaria who received the Fe chelator desferrioxamine, which corresponded to antimalarial immune responses. In Africa, an endemic form of secondary Fe overload, caused by the consumption of traditional Fe-containing beer and linked to the presence of a mutation in the ferroportin

gene (Gordeuk et al. 2003), is associated with an increased incidence of and mortality from tuberculosis. These latter data are in accordance with *in vitro* findings, which find that changes in intramacrophage Fe availability stimulates the proliferation of mycobacteria and weakens the antimycobacterial defense mechanisms of macrophages. Other infections—ranging from bacterial, viral, and fungal diseases to parasitic diseases, where Fe overload is associated with an unfavorable course of the infection and/or an impaired immune response—have been well summarized (Weinberg 1999).

The importance of iron for the antimicrobial immune response pathways is further underscored by the finding that many innate resistance genes of macrophages act by limiting Fe availability for intracellular bacteria. Macrophages exposed to the intracellular bacterium *S. typhimurium* increase the expression of the Fe export protein ferroportin; this stimulates cellular Fe export and limits Fe availability for intramacrophage bacteria, leading to improved control of bacterial proliferation by macrophages. In part, this is because a reduction of cytoplasmic iron increases the activity of immune effector pathways, such as TNF-α, IL-6, IL-12, or NO formation (Nairz et al. 2010). Importantly, part of the antimicrobial activities of the Th-1 cytokine IFN-γ and iNOS have been linked to their ability to limit Fe availability in bacteria. Briefly, induction of iNOS by macrophages exposed to intracellular bacteria results in an activation of the transcription factor Nrf2; this stimulates ferroportin transcription, induces the export of iron from *Salmonella*-infected macrophages, and stimulates antimicrobial immune effector pathways. Importantly, the impaired control of *Salmonella* infection in iNOS–/– mice can be completely overcome by treatment with the Fe chelator desferrasirox (Nairz et al. 2013). The crucial role of ferroportin-mediated Fe export for host defense against infections with intracellular pathogens is further supported by the observation that overexpression of ferroportin in macrophages can control the infection with a number of intracellular bacteria, such as *Chlamydia* spp., *Legionella*, *Salmonella*, or *Mycobacteria* (Nairz et al. 2010).

Another immune gene that exemplifies the role of iron in infection is the phagolysosomal protein NRAMP1. The expression of Nramp1 is associated with resistance toward infections in intracellular pathogens such as *Leishmania*, *Salmonella*, or *Mycobacteria* spp., and occurs mainly by shuttling divalent metals across the phagolysomal membrane (Forbes and Gros 2001; Blackwell et al. 2001). Investigations of the RAW264.7 macrophage cell line stably transfected with functional or nonfunctional Nramp1 have demonstrated that macrophages expressing functional Nramp1 exhibit significantly lower iron uptake via TfR and increased Fe release mediated through increased ferroportin expression. Accordingly, as a net effect of the altered expression of Fe transporters, the overall cellular Fe content was lower in macrophages bearing functional Nramp1 (Fritsche et al. 2012). This provides further support for the hypothesis that NRAMP1 expression confers resistance toward intracellular pathogens by limiting the availability of iron to the microbes; a contribution

to Fe-mediated formation of toxic radicals has also been discussed (Forbes and Gros 2001). In addition, NRAMP1-mediated alterations of Fe homeostasis stimulate antimicrobial immune effector function, as evidenced by increased formation of NO or TNF-α, whereas expression of the anti-inflammatory cytokine IL-10 is significantly reduced (Nairz et al. 2010). Recent evidence suggests that Nramp1 functionality results in increased formation of lipocalin 2 (NGAL, Lcn2) (Fritsche et al. 2012). Lipocalin 2 is a neutrophil and macrophage-derived peptide that captures Fe-laden microbial siderophores, thus interfering with the acquisition of siderophore-bound iron by specific Gram-negative bacteria, such as *Escherichia coli* or *Klebsiella* spp. (Flo et al. 2004). Moreover, Lcn2 delivers siderophore-bound iron to mammalian cells, which are able to import the complex via Lcn2 receptor. Most interestingly, recent data provide evidence for the existence of mammalian siderophores that are captured by Lcn2, indicating that Lcn2 may be involved in transcellular and transmembrane Fe trafficking in mammals (Pantopoulos et al. 2012).

In addition, Lcn2 expression affects neutrophil recruitment to the sites of infection which, depending on the underlying pathogen, exerts contrasting effects on the outcome of the infection (Warszawska et al. 2013). Interestingly, Lcn2 is also secreted during infection with non-siderophore-producing pathogens, such as *Chlamydia* or *Plasmodia*. This limits Fe availability for *Plasmodia* and impairs erythropoiesis, which further inhibits replication of plasmodia. In addition, following the mechanisms described above, Lcn2 stimulates innate immune responses by limiting Fe availability (Zhao et al. 2012). Similarly, lipocalin 2 may confer resistance to infection by *Salmonella* and *Mycobacteria* spp. in patients suffering from hereditary hemochromatosis. As a consequence of reduced hepcidin formation and increased expression of Lcn2 upon infection, macrophages of these patients are Fe deficient, thus constituting a hostile environment for these pathogens (Nairz et al. 2009).

It is important to note that host-mediated alterations of Fe homeostasis (e.g., via the formation of hepcidin) may directly impact the proliferation of microbes. Hepcidin expression has been shown to affect the proliferation of intrahepatic sporozoites negatively, but it may also increase susceptibility to infection with Fe-dependent pathogens such as *Salmonella* (Portugal et al. 2011). It appears that different regulatory mechanisms are initiated, depending on the type of the infectious pathogens. Although hepcidin induction appears to be a very efficient strategy to limit the availability of iron for extracellular bacteria, since it restricts the nutrient iron within the reticuloendothelial system, this strategy may be detrimental to intracellular pathogens. For intracellular pathogens, multiple pathways (e.g., NOS2, ferroportin formation, NRAMP1) lead to mobilization and export of iron out of macrophages, rendering them Fe deficient; this ameliorates the control of infection with intracellular microbes and positively affects innate immune responses.

Alterations of Copper, Zinc, and Manganese Homeostasis during Infection

Copper homeostasis is closely linked to Fe metabolism, because the ferroxidases hephaestin and ceruloplasmin (which mediate the oxidation of ferrous to ferric iron and its incorporation into transferrin) are Cu-containing enzymes (Pantopoulos et al. 2012). Thus, Cu deficiency leads to Fe overload and subsequent Fe-mediated tissue damage. In addition, copper plays important roles as a prosthetic group for many enzymes, such as cytochromes, proteins involved in oxidative phosphorylation, and Cu or Zn superoxide dismutase (Hood and Skaar 2012).

As with iron, copper is a redox-active metal iron that is able to catalyze the formation of toxic hydroxyl radicals; Cu accumulation is thus associated with increased antimicrobial toxicity, also termed metal poisoning (Samanovic et al. 2012). Evidence suggests that copper kills microbes via mechanisms that are independent from radical formation (e.g., by displacing iron from Fe-S clusters within enzymes); however, further investigation into these functions is needed. Of note, copper and zinc have been shown to accumulate in phagosomes of macrophages infected with *M. tuberculosis* (Wagner et al. 2005). Proinflammatory cytokines, such as IFN-γ, induce the expression of the Cu permease Ctr1 in macrophages, which results in an increased Cu uptake into macrophages as well as translocation of the P-type ATPase ATP7A to phagolysosomes. This mediates Cu influx into these vesicles and subsequent metal poisoning of bacteria (White et al. 2009). Copper deficiency is, therefore, associated with an increased susceptibility to infections.

The transition metal zinc plays a central role in the function of structural proteins and is essential for immune cell proliferation and differentiation (Liu et al. 2012). Cell differentiation is manifested through the association between Zn deficiency and thymic atrophy, impaired B, T, and NK cell responses, as well as reduced formation of proinflammatory cytokines. Zinc, however, can be used as an antimicrobial weapon to intoxicate microbes. Recent evidence suggests that the granulocyte macrophage colony stimulating factor (GM-CSF) induces the sequestration of zinc in macrophages. Specifically, GM-CSF induced the expression of two Zn transport proteins, leading to accumulation of the metal in the Golgi apparatus which triggered the formation of toxic radicals by virtue of NADPH oxidase, thereby exerting antifungal activity against *Histoplasma capsulatum* (Vignesh et al. 2013a).

Of interest, bacteria need zinc for their pathogenicity and as part of their defense against host-mediated oxidative stress. In randomized controlled trials aimed at improving children's health by avoiding negative effects of Fe and Zn deficiency on growth and mental development, daily dietary supplementation of iron and zinc, in the form of micro-powders, resulted in an increased burden of infections (Soofi et al. 2013). Therefore, Zn sequestration during an infection is considered to represent another branch of nutritional immunity. Components

of the S100 protein family bind Zn^{+2}, Cu^{+2}, and Mn^{+2} (see below), which exert antimicrobial activity (Hood and Skaar 2012). Two of these proteins, S100A8 and S100A9, form a heterodimeric complex named calprotectin. Calprotectin is expressed by neutrophils, where it accounts for up to 50% of cytoplasmic proteins. The secretion of calprotectin is performed primarily by apoptotic neutrophils and associated with extracellular traps. Binding and sequestration of zinc and manganese by calprotectin is suggested to exert antimicrobial activity by weakening the antioxidant defense mechanism of microbes, which need these two molecules to activate their radical detoxifying enzymes. In addition, other S100 proteins, such as S100A12 (calcitermin), can also bind transition metals. Found in human airways, S100A12 exerts antimicrobial activity against several bacteria as well as against fungi or nematodes. The expression of S100 proteins can be induced by IL-17 and IL-22, whereas S100 proteins influence immune function by exerting proinflammatory activity and promoting neutrophil chemotaxis (Hood and Skaar 2012). The S100A7 protein (also termed psoriasin) is secreted by keratinocytes where it exerts antimicrobial actions after binding with zinc. It is important to bear in mind, however, that metal depletion strategies of the host affect pathogenic bacteria as well as the commensal or protective flora. Thus, bacteria which have evolved strategies to outcompete these metal restrictions benefit from a developmental advantage and may thus become more pathogenic (Liu et al. 2012).

As briefly mentioned, Zn sequestration is often accompanied by the capture of manganese, which is needed by microbes as part of the antioxidant defense protein Mn or Zn superoxide dismutase and as a catalytic component of several central proteins (where it can also replace the more redox active metal iron). Like iron, both zinc and manganese are transported by NRAMP1, and limitation of Mn availability within the phagolysosome is considered to be an important mechanism by which macrophages confer resistance toward infection with intracellular pathogens. In addition, NRAMP2 (better known as DMT1) transports a myriad of divalent metal ions across membranes in an ATP- and proton-dependent process. DMT1 expression and Fe transport capacity are increased in inflammatory macrophages (Ludwiczek et al. 2003); however, it has not been investigated thus far whether this is also paralleled by increased accumulation of copper, zinc, or manganese in macrophages and whether or not this strengthens antimicrobial activities. The central role of Mn starvation in antimicrobial activity has recently been underpinned by the finding that calprotectin-mediated Mn restriction causes maximum growth inhibition of bacteria (Damo et al. 2013).

Conclusion

Transition metals play decisive roles in host–pathogen interactions and affect the outcome of infections, due to their essential nature in pathogen proliferation

and microbial resistance mechanisms to oxidative stress. In addition, these metals are important for host immune cell proliferation and differentiation, as well as for mounting an effective antimicrobial immune response.

More efforts are needed to understand metal trafficking between the host and microbe. Specifically, we need to disentangle the multiple roles of these transition metals for innate and adaptive antimicrobial immune responses as well as for microbes. Thus far, most studies have concentrated on evaluating the role of a single metal in host–pathogen interactions, yet the metabolisms of different transition metals are interconnected in multiple ways. To gain new insights into the cross-regulatory interactions of infection, and ultimately *in vivo*, the effects of metals like iron, zinc, copper, and manganese need to be evaluated in parallel in host–pathogen interactions. The resulting knowledge will enable novel therapeutic targets for tackling microbes to be identified and provide insight into how modulation of metal metabolism, on either the host or pathogen site, can positively affect the course of infections.