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Metals in Host–Microbe Interaction

The Host Perspective

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Abstract

This overview covers the role of the metal ions in infectious diseases, focusing on iron (Fe), copper (Cu), zinc (Zn), and, to a lesser extent, manganese (Mn) and the metalloid selenium (Se). In addition, recommended dietary allowances are addressed, as are metal-based drugs for the treatment of tropical diseases.

The human organism binds essential metals such as iron, manganese, copper, and zinc to specific compounds (including proteins) in order to withhold these metals from invading pathogens ("nutritional immunity"); in this way, metal binding provides resistance to infection. Selenium status can also affect the host–pathogen interaction, but pathogens have mechanisms to counteract this protective potency. As alternative to a withdrawal of metals, microbes can be exposed to particularly high—and thus toxic—levels of metal ions. A secondary protective mechanism stems from the production (by host innate immune cells) of reactive oxygen and nitrogen species; this can also result in host tissue damage. In addition, the gasotransmitters nitric oxide (an oxidant) and carbon monoxide are indirectly involved in side effects (deprotection and protection, respectively, of bound heme) that result from the immune response.

Host-mediated alteration of Fe homeostasis directly impacts on the proliferation of microbes. Depending on the type of pathogen, different regulatory mechanisms can be initiated. Limiting the availability of iron can be an efficient strategy to restrict extracellular bacteria, although such a strategy is detrimental for intracellular pathogens. Iron homeostasis is partly linked to Cu homeostasis. Copper deficiency predisposes mammals to infectious diseases, to some extent as a consequence of a lack of neutrophils induced by inadequate Cu availability or supply. Finally, there is a clear-cut cor-

relation between bacterial infections and Zn removal from serum. More generally, Zn deficiency reduces immune defense against infections, chronic inflammatory disease, and reduced cellular activation, whereas high levels of zinc can hamper effective signal transduction.

Due to the epidemic proportions of tropical diseases (e.g., leishmaniasis, Chagas disease, and malaria) and lack of effective treatment, drugs are being developed that are based on coordination compounds of metals, including copper, iron, ruthenium, and gold. These metals are coordinated to aromatic ligand systems that allow for a stabilization of the drug, during the drug's transport to its target, and eventually intercalation into DNA. For malaria, the increasing resistance of the malaria parasite against the classical drug chloroquine may be overcome by employing ferrocenyl derivatives of chloroquine.

Introduction

This report focuses on the distribution and function of the essential elements iron, zinc, copper and, to some extent, manganese, and selenium. Molybdenum was excluded because its impact on infectious diseases is hardly known. Also excluded were vanadium, chromium, nickel, and cobalt. Chromium has long been considered to be an indispensable trace metal (Mertz 1993), but from today's point of view, it is unlikely to be essential (Cefalu et al. 2010); there is, however, evidence of its potential to invoke cancer in the +V state, generated from chromate(VI) under physiological conditions (Levina et al. 2009). The essentiality of vanadium has not yet been established. The similarity, however, between vanadate and phosphate strongly suggest a role for vanadate in the regulation of phosphate-metabolizing enzymes, such as phosphatases and kinases (Rehder 2014). An example is the antidiabetic effect of vanadate as a consequence of the inhibition of protein-tyrosine-phosphatase through the coordination of vanadate to the cysteinate in the active site. Cobalt is the central metal in vitamin B12; however, whether external "free" cobalt is a nutritional requirement has yet to be clarified. Finally, nickel does not appear to be directly essential for humans, yet its inalienability for microbes—and thus also for the human microbiome—presupposes an indirect essentiality also for humans. More generally, the consequences of the microbiome for metal availability in the host, and vice versa, remain to be explored.

The essentiality of many transition metals, and of the main group element selenium, presupposes that these elements are available as micronutrients for humans as well as for both symbiotic and pathogenic microbes and parasitic protozoa. In response to an infection, the host can increase the metal concentration up to levels that are toxic for the pathogen or reduce the availability of the essential metal for the invading microbes, using specific metal ion-binding proteins and/or by withdrawing metal ions from affected cells and tissues. On the other hand, parasitic microorganisms have developed sophisticated mechanisms to cope with metal ion restrictions (Diaz-Ochoda et al. 2014) and overloads.

We begin with an overview of biologically essential elements, in general, and metals/metalloids that are essential for humans and of interest in the host–microbe interaction. We address in some detail the distribution and function of manganese, iron, copper, zinc, and selenium, emphasizing the competition for these elements in the frame of the interaction between the host (the human body) and the invading microbes/parasites. The role of the gasotransmitters carbon monoxide (CO) and nitric oxide (NO) is presented in relation to their interference with bound and free ferrous ions, followed by a brief discussion on dietary allowances for essential metals, as recommended by the World Health Organization (WHO). Finally, we conclude with a discussion of metal-based drugs in the treatment of tropical diseases caused by parasitic protozoa.

Essential Metals and Metalloids: An Overview

Figure 13.1 shows the periodic table of life elements. To ascertain that neither deficiency nor overload occurs, metal homeostasis must be tightly regulated. Even in the case of intact metal homeostasis, deficiencies are inevitable when a person is subjected to an unbalanced diet. This happens, in particular, in infants, children, pregnant and nursing women, as well as elderly individuals. People who are particularly susceptible to an unbalanced provision (commonly an undersupply) of essential metals and metalloids are those at risk

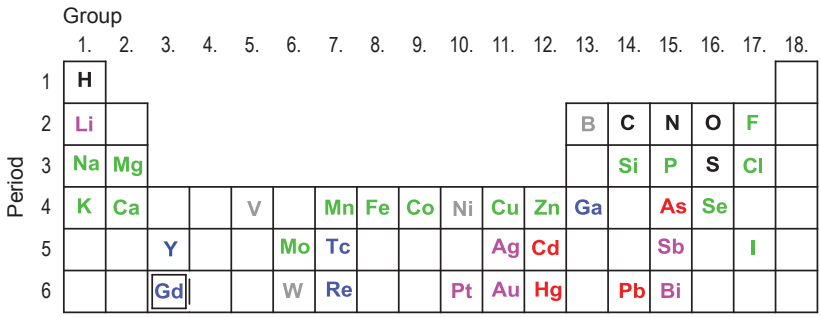

Figure 13.1 Biologically and medicinally relevant elements. Elements that are demonstrably essential for humans are shown in black (for the buildup of organic compounds) and green. Elements that are essential for only select groups of organisms are noted in gray; toxic metals are listed in red. Metals used in therapy are shown in mauve: Li (bipolar disorder), Ag (wound disinfection), Sb (leishmaniasis, schistosomiasis), Pt (cancer therapy), Au (rheumatoid arthritis), Bi (gastrointestinal disorders). Metals employed in diagnostic techniques (such as positron emission tomography, PET, and magnetic resonance imaging, MRI) are highlighted in blue. Gd is framed, because it is an f-group element. Other metal prescriptions are sporadically used medicinally. For example, lanthanum carbonate is sometimes applied in dialysis to reduce serum phosphate levels (hyperphosphatemia). The essentiality of chromium (e.g., in the form of a hardly defined picolinato-Cr^{3+} complex, the "glucose tolerance factor") has recently been critically scrutinized (Cefalu et al. 2010); Cr has thus not been included.

of undernourishment and/or malnutrition, such as inhabitants of developing countries, people with infections, and, to some extent, vegans.

Recommended dietary allowances (RDAs) for metals, as released and recommended by governmental authorities and the WHO, need to be reviewed and assessed critically in light of the scientific arguments presented in this publication; for detailed discussion of RDAs and relevant tables, see Wang and Zhang (this volume). One problem with RDAs is that speciation (and thus bioavailability) of the respective nutritional element is not considered. Open questions include adverse effects from normal levels of metal supplementation (e.g., iron in relation to copper), in particular with respect to long-term effects, dietary needs, infections, and chronic diseases. Eventually, in setting (upper) limits for the intake of essential metals, differentiation by population should be considered.

Below we provide an overview of the uptake, distribution, and *selected* specific functions of elements essential for humans. Further details on manganese, iron, zinc, and copper, and the metalloid selenium in a broader context are provided by Wessels and Loutet et al. (this volume).

Magnesium

Magnesium plays a central role in phosphate (and hence energy) metabolism. For example, Mg adenosine triphosphate (ATP)—where Mg^{2+} is coordinated to phosphate, H_2O/OH^-, and the carboxylate of Asp or Glu—activates the phosphate-dependent metabolic pathways. Magnesium provides support to endoskeletons, and stabilizes the structure of proteins and polysaccharides.

Calcium

The majority (about 99%) of calcium is present in bones as hydroxyapatite $Ca_5(PO_4)_3(OH)_{1-x}F_x$ ($x \leq 0.01$) and teeth ($x \sim 0.1$). Calcium also plays a role in muscle contraction/relaxation, blood clotting, enzyme regulation, the gating of K^+ channels, stabilization of protein structures, and signal transduction. Extracellular $c(Ca^{2+})$ exceeds intracellular $c(Ca^{2+})$ by a factor of 2.5×10^3. Three hormones are involved in Ca^{2+} metabolism.

Manganese

Manganese is associated with the lipid, carbohydrate, and amino acid metabolism. Examples of Mn-dependent enzymes in humans are prolidase, arginase, and mitochondrial superoxide dismutase (SOD). The Ca^{2+}-binding protein calprotectin also effectively binds Mn^{2+}. Mn^{2+} and Zn^{2+} share some chemical properties; there is, however, no established connection for the competition between these two metal ions.

Iron

Iron is the most abundant transition metal in the human body. It is critically involved in key physiologic functions, including the transport of gaseous molecules, such as oxygen O_2 (hemoglobin), or gasotransmitters, such as NO and CO, electron transport in the mitochodria, and activity of a variety of redox enzymes. Redox enzymes include many that are involved in the formation of free radicals implicated in host defense mechanisms that target pathogens for destruction (e.g., the Fenton reaction). These are commonly based either on heme iron or on iron-sulfur (Fe-S) proteins.

Most iron in the body is recycled; a daily uptake of 1–2 mg suffices for maintenance of Fe homeostasis. A schematic of Fe uptake, distribution, and recycling is illustrated in Figure 13.2. It should be noted that only 20–25% of the bioavailable iron in mammals exists in the form of accessible iron, bound to transferrin, stored inside ferritin, in Fe-S clusters or transiently bound to Fe chaperones and transporters. The remaining 75–80% of bioavailable iron exists inside the protoporphyrine IX ring of heme, the prosthetic group of hemoproteins. Among these, hemoglobin comprises up to 70% of the total pool of heme, thus accounting for the major Fe compartment in mammals. Bioavailable iron can transit from the heme into the labile Fe compartment via a controlled process that relies on heme catabolism by heme oxygenases, as illustrated by the finding that the heme oxygenase-1 isoform, constitutively expressed by hemophagocytic macrophages, is essential to maintain Fe homeostasis (Poss and Tonegawa 1997; Yachie et al. 1999). It becomes apparent therefore that regulation of heme catabolism, allowing for the extraction and recycling of iron into the labile Fe compartment, is essential for the maintenance of Fe homeostasis

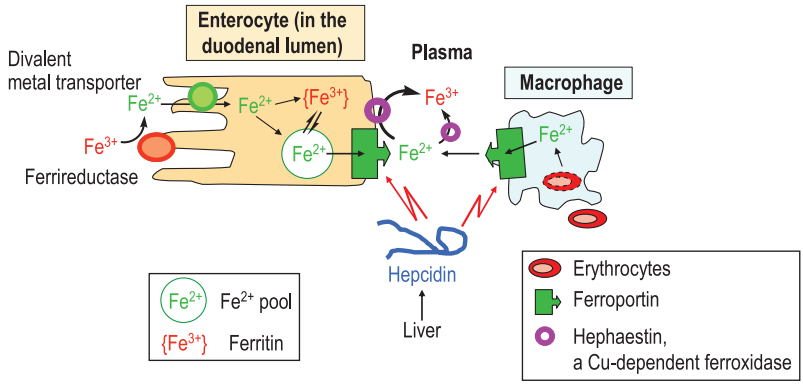

Figure 13.2 Uptake of iron and Fe homeostasis. In blood plasma, ferric iron is tightly bound to the transport protein transferrin; ca. 80% of the overall iron is present in the form of heme. The intestinal absorptive cells, termed enterocytes, are located in the duodenal lumen. Hepcidin, a peptide hormone produced in the liver, attains a central role due to its binding to the Fe transporter ferroportin, which *decreases* Fe flow into the plasma by initiating endocytosis and proteolysis of ferroportin.

in mammals (Gozzelino and Soares 2014). Maintenance of Fe homeostasis also relies on the transit of labile iron into the Fe-heme compartment, through de novo heme synthesis, catalyzed by a sequence of eight enzymatic steps completed by the insertion of iron into the proptoporphyrin IX ring, and catalyzed by ferrochelatase. This is essential, for example, for the insertion of Fe-heme into hemoglobin and maintenance of erythropoiesis.

An inadequate supply of iron can lead to anemia and Friedreich ataxia, whereas abundance can result in oxidative stress (due to abnormal Fe deposition) and hemochromatosis.

Cobalt

Cobalt is the central metal ion (Co^{+}, Co^{2+}, Co^{3+}) of vitamin B12 and its derivatives. An undersupply of cobalt causes pernicious anemia and results, for example, in damage to nervous tissues. Larger doses of cobalt are acutely toxic. "Free" cobalt, supplied through food, cannot be used in the human physiological system.

Copper

Copper is present in various redox enzymes, where the metal switches between the oxidation states +I and +II. Copper is also involved in Fe homeostasis (see, e.g., hephaestin in Figure 13.2). Several diseases are connected to Cu imbalance: Wilson disease (Cu overload), Menkes disease (Cu deficiency) and possibly Alzheimer disease (imbalance between "labile" and "tightly bound" Cu). In grazing cattle, molybdate MoO_4^{2-} in soils can immobilize copper and lead to Cu deficiency.

Zinc

Zinc (Zn^{2+}; redox inactive under physiological conditions) is the second most abundant transition metal in the human body and forms the active center of enzymes, including hydrolases, carboanhydrase, and alcohol dehydrogenase. Other Zn-dependent functions are manifest in genetic transcription ("Zn fingers"), in the stabilization of tertiary and quaternary structures of peptides, and in DNA repair proteins. Low molecular mass proteins rich in zinc, the thioneins, store zinc and regulate Zn levels, but can also act as scavengers for toxic Pb^{2+}, Cd^{2+} and Hg^{2+} (for an updated view of zinc, see Chasapis et al. 2012).

Molybdenum

Molybdenum is a constituent of the molybdopterin cofactor in three enzymes in humans: sulfite oxidase (SuOx), xanthine oxidase (XaOx), and DMSO reductase. SuOx is located in the mitochondria, where it catalyzes the oxidation—and

thus detoxification—of sulfite (supplied exogenously, or formed in the frame of the metabolism of cysteine and methionine) to sulfate. An inadequate supply of molybdenum causes severe neurological damage in early childhood. XaOx catalyzes the oxidation/dehydrogenation of aldehydes as well as the oxidation of hypoxanthine to xanthine. Dimethyl sulfoxide reductase catalyzes the reduction of dimethyl sulfoxide to dimethylsulfide.

Selenium

Selenium is an essential micronutrient, present in the body in the form of selenocysteine SeCys and selenomethionine. SeCys serves as a building block in enzymes, such as glutathione peroxidase, and in some molybdopterin cofactors.

Toxic Elements

The toxicity of cadmium, mercury, and lead results from the ability of these metal ions to form very stable thiocompounds, and thus to denature peptides and proteins through the reaction with cysteine sulfhydryl groups. In addition, Zn metabolism is disturbed when Zn^{2+} is replaced in Zn thioneins. Nickel and aluminum are other possibly nonessential elements that can disrupt metabolic pathways when present in more than trace amounts.

The Role of Manganese, Iron, Copper, Zinc, and Selenium

Manganese

Innate Immunity

Similar to iron and zinc, mammals bind manganese in an attempt to withhold this transition metal from invading pathogens. The neutrophil-derived protein, calprotectin, chelates both zinc and manganese, removing these metals from sites of infection. Calprotectin is a heterodimer encoded by the genes *S100A8* and *S100A9*, and is one of the most abundant proteins at sites of inflammation. While the relative contribution of Zn and Mn chelation to the inhibition of infections remains to be completely defined, it is clear that Mn chelation by calprotectin is required to inhibit bacterial growth to the greatest extent possible (Corbin et al. 2008) and, as such, to provide resistance to bacterial infections. Moreover, Zn and Mn chelation can inhibit microbial virulence factors that rely on these metals (Liu et al. 2012). The protein S100A12 is also constitutively expressed in neutrophils and has a role in infection and immunity, presumably through Zn binding (Goyette and Geczy 2011). In addition, S100A7 inhibits microbial growth through Zn binding and has an important role in protecting against infections of the skin (Schröder and Harder 2006). Notably, the S100 protein family is comprised of 24 family members,

including many metal-binding proteins. This raises the exciting possibility that additional Mn- and Zn-binding components of the innate immune system have yet to be uncovered. In an interesting turn of events, *Neisseria meningitidis*, the causative agent of meningitis, has been reported to produce a protein that binds calprotectin and thus enables the organism to utilize this protein as a nutrient Zn source (Stork et al. 2013). It is likely that bacteria have multiple strategies to circumvent calprotectin-dependent metal binding, but these strategies have yet to be uncovered.

Transport and Inflammation

Currently, undefined mammalian compound(s) contribute to Mn chelation. Although some studies have detected manganese bound to transferrin and lactoferrin (Aschner and Gannon 1994), their contribution to withholding manganese from pathogens has not been determined. Mutation of Mn uptake systems in a number of bacterial pathogens results in a loss of virulence in various pathogenesis models (Porcheron et al. 2013). This is further support that chelation of manganese in mammals creates a Mn-deficient environment which pathogens must overcome.

Due to the ubiquitous occurrence of manganese in the diet, daily dietary uptake in industrial countries is higher than the recommended daily requirement. Thus Mn deficiency is extremely rare and has only been shown in experimental models (Roth 2006). Deficiency has been demonstrated in several animal species (e.g., rats, mice, pigs, chicken, and cattle) and can result in several biochemical and structural defects such as poor bone growth, skeletal abnormalities, ataxia, and abnormal glucose tolerance (Santamaria and Sulsky 2010). In human subjects on an experimental low Mn diet, dermatitis, slowed growth of hair and nails, decreased serum cholesterol levels, and decreased levels of clotting proteins have been reported (Finley et al. 2003).

In contrast, toxicity is more common, and chronic manganese overexposure has been shown to correlate with permanently progressing neurodegenerative damage. In a few studies that targeted neuropathological mechanisms, the role of neuroinflammation has been uncovered: Manganese enhances the release of the inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor (TNF) from resident macrophages in the brain, i.e., microglial cells which can promote the activation of astrocytes and subsequent release of inflammatory mediators such as prostaglandin E2 and the gasotransmitter NO (Aschner et al. 2009; Filipov and Dodd 2011). In addition, manganese potentiates NO production in cytokine-stimulated astrocytes (Liu et al. 2005).

In biological systems, the most relevant and stable Mn species exist in the form of Mn^{2+} and Mn^{3+}. These Mn ions are transported into cells via several mechanisms (e.g., facilitated diffusion and active transport) and transporters for other divalent ions (e.g., Fe^{2+} and Zn^{2+}), indicating an interconnection between Mn, Fe, and Zn homeostasis. Manganese is discussed to be transported,

among others, via the divalent metal transporter 1 (DMT1), the transferrin receptor (TfR), the divalent metal/bicarbonate ion symporters ZIP8 and ZIP14, various calcium channels, the solute carrier-39 (SLC39) family of Zn transporters, park9/ATP13A2, the Mg transporter hip14, the transient receptor potential melastatin 7 (TRPM7) channels/transporters, homomeric purine receptors (P2X and P2Y), and the citrate transporter (Bowman et al. 2011).

Iron

Heme Iron in Host–Microbe Interaction

There is a key and singular aspect related to Fe metabolism that should be taken into consideration in the context of host–microbe interactions in mammals: the vast majority of bioavailable iron in the affected host is actually contained inside the prosthetic heme groups of hemoproteins. Mechanisms which control host heme synthesis, transport (Yuan et al. 2013), and catabolism (Gozzelino and Soares 2014) should thus play a critical role in the maintenance of host–microbe interactions and in the pathologic outcome of infectious diseases.

Reactive oxygen and nitrogen species produced by host innate immune cells during microbial infections, which confers resistance to infection, can eventually lead to host tissue damage and exacerbate rather than prevent the severity of infectious diseases (Medzhitov et al. 2012). Moreover, inflammatory and immune responses are associated with varying degrees of hemolysis and rhabdomyolysis, and hence with the release of noncovalently bound heme from hemoglobin and myoglobin, generating free heme (Gozzelino et al. 2012). The impact of this event in the deregulation of Fe metabolism during infections should be appreciated, taking into account that the majority of the iron is contained within the heme groups of hemoglobin and myoglobin (Gozzelino and Soares 2014). The redox activity of nonhemoprotein-bound heme, "free heme," is no longer under the control of the amino acids that surround the prosthetic heme groups within the so-called heme pockets of these hemoproteins. Presumably for this reason, free heme is highly pro-oxidant and cytotoxic, exerting proinflammatory effects through the engagement of specific pattern recognition receptors, such as the Toll-like receptor 4 (TLR-4) that is expressed by monocytes/macrophages as well as in endothelial cells (Belcher et al. 2014). Free heme can also exert chemotactic effects via the engagement of G protein-coupled receptors expressed in polymorphonucelar cells. The combination of these effects has a significant and yet unappreciated impact on the outcome of infectious diseases (Gozzelino et al. 2012).

To ensure regulated adaptation of heme transport and catabolism, several stress response systems have evolved to minimize the negative impact caused by the generation of free heme during infection (Gozzelino et al. 2010). These systems are essential to control the proinflammatory, vasoactive and cytotoxicity effects of heme, as well as to sustain systemic Fe homeostasis, avoiding

tissue Fe overload, tissue damage, and anemia in the infected host (Soares et al. 2009). The impact of such regulatory pathways has been demonstrated unequivocally for the heme-catabolizing enzyme heme oxygenase-1 (HO-1) in mice, as well as in humans (Yachie et al. 1999), where HO-1 deficiency is associated with impaired Fe recycling, anemia, vascular damage, and depletion of hemophagocytic macrophages (Kovtunovych et al. 2010), presumably driven by heme cytotoxicity (Fortes et al. 2012). Moreover HO-1 deficiency renders infected hosts extremely susceptible to systemic infections, as demonstrated for severe sepsis (Larsen et al. 2012), malaria, and more recently for tuberculosis (Silva-Gomes et al. 2013).

The protective effect afforded by HO-1 expression against systemic infections has been linked to the adaptation of cells to cellular and tissue Fe overload (Gozzelino and Soares 2014). This is demonstrated by the finding that deletion of the ferritin H chain gene, which acts downstream of HO-1 to neutralize the pro-oxidant effect of the iron released from heme catabolism, is strictly required to support the protective effect of HO-1 against systemic infections, as has been demonstrated for malaria.

In the context of systemic infections, the induction of HO-1 expression in the host is essential to provide protection against the cytotoxic effects and presumably the proinflammatory and vasoactive impact of free heme. These salutary effects are essential to reduce tissue damage and hence disease severity, and to support host survival. This host defense strategy, while essential to the survival of an infected host, does not appear to exert a negative impact on the pathogen—a phenomenon referred to as disease tolerance (Gozzelino and Soares 2014).

Gasotransmitters: The Interaction of Nitric Oxide and Carbon Monoxide with the Heme Group

Gasotransmitters are biologically active molecules produced physiologically by several evolutionary conserved enzymes (Mustafa et al. 2009). These gaseous molecules include nitric oxide, generated by the enzymatic conversion of L-arginine and molecular oxygen to L-citrulline (Figure 13.3a) and catalyzed by different nitric oxide synthases (NOS). Carbon monoxide is generated through the catabolism of heme (Figure 13.3b) and catalyzed by different isoforms of heme oxidases. Hydrogen sulfide (H_2S) is produced from cysteine by cystathionine beta-synthase and cystathionine gamma-lyase, but will not be covered in further detail here.

Gasotransmitters share biophysical properties that include lipid solubility, which allows for diffusion across cellular membranes and underlies their intrinsic ability to target, more or less distally, specific intracellular signal transduction pathways without requiring classical cognate ligand/receptor interactions (Mustafa et al. 2009). This occurs, for example, via the physical interaction of gaseous molecules with transition metals (Cooper 1999). This is

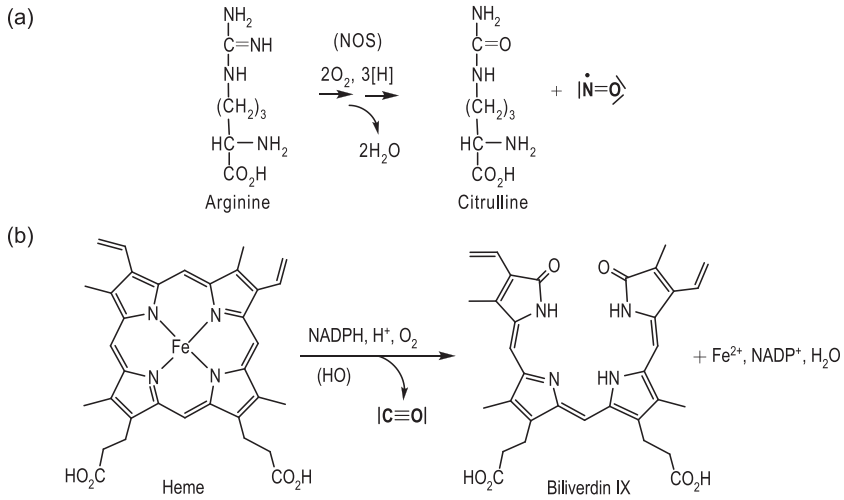

Figure 13.3 (a) Formation of nitric oxide: NO is catalyzed by nitrogen oxide synthase, NOS, in a two-step process via an [NOH] intermediate. [H] represents reduction equivalents, commonly delivered by nicotine adenine dinucleotide NADPH. (b) Carbon monoxide (CO) forms by oxidative catabolism of free heme, catalyzed by heme oxygenase (HO). A potent inducer for the transcription of the HO-1 gene is nitric oxide.

well illustrated for iron in the context of Fe-S clusters or heme groups within metalloproteins. Briefly, NO or CO binding to Fe^{2+} within Fe-S clusters or in the prosthetic heme groups of metalloproteins can modify the tertiary structure of those proteins and their biological activity, as illustrated, among others, for guanylate cyclase, an enzyme that generates cyclic guanosine monophosphate (cGMP), or for cytochrome-c. Although the signaling pathway for NO includes other well-established mechanisms (e.g., tyrosine nitrosylation), transition metal-mediated signal transduction appears to be a general principle by which gasotransmitters exert their biological effects. While the output of this interaction is specific to each gasotransmitter, this common mechanism illustrates how regulation of the metabolism of transition metals may affect the biologic action of gasotransmitters.

Interaction of gasotransmitters with Fe^{2+} produces additional effects that can be critical for the outcome of infectious diseases. For example, as a prooxidant labile-free radical, NO's interaction with Fe^{2+} in the heme group of hemoglobin results not only in tight binding and scavenging of this gasotransmitter, but also promotes the oxidation of hemoglobin (Cooper 1999); this, in turn, can then act in a proinflammatory manner (Silva et al. 2009) to impact the outcome of infectious diseases, as illustrated for sepsis (Larsen et al. 2010; Adamzik et al. 2012). In contrast, the interaction of CO with Fe^{2+} in the heme groups of hemoglobin has the opposite effect: Fe heme oxidation is prevented as is hemoglobin oxidation. Ultimately, this blocks heme release from the

globulin chains of hemoglobin (Pamplona et al. 2007; Gozzelino et al. 2010). This latter effect is sufficient in itself to explain the protective effect of this gasotransmitter against the pathological outcome of malaria (Pamplona et al. 2007; Ferreira et al. 2008), the infectious disease triggered by *Plasmodium* infection (Miller et al. 2013). It is likely that in a similar manner to hemoglobin, CO can prevent heme release from other hemoproteins such as myoglobin. Whether this effect of CO also limits the pathologic outcome of other infectious diseases remains to be established, but it is likely to be the case.

Interplay of Iron Distribution between (Infectious) Microbes and Host Cells

Infected host cells tend to control the homeostasis of essential metals, either by limiting the availability of metals that are necessary to maintain the physiology and replication of microbes, or by exposing microbes to high (and thus toxic) concentrations of metals directly or indirectly (e.g., by increasing free radical formation). One example is the Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$. The host immune system affects the availability of essential metal ions for microbes through cytokines (hormonal regulators or signaling molecules). The microbes, in turn, activate pathways to secure a sufficient supply of metal ions.

In the case of Fe homeostasis (cf. discussion in the next section), monocytes and differentiated macrophages (i.e., cells that digest bacteria, dying cells, and senescent erythrocytes) are involved in the regulation of Fe levels and free radical production, including reactive oxygen and nitrogen species. The limitation of the availability of iron for invading pathogens is termed "nutritional immunity." Instantaneous Fe retention by macrophages (and thus restricted availability of plasma iron in the case of an infection) is also induced through binding greater amounts of hepcidin to the ferroportin (Figure 13.2) of macrophages; this switches off the transport protein ferroportin and causes Fe retention in the macrophages and Fe depletion in the (infected) surroundings. In addition, decreased expression of ferroportin, coupled with the inhibition of ferritin (the Fe storage protein) translation, provides a basic means to reduce Fe supply. Inhibition of ferritin translation, in turn, is partly coupled to the stimulation of NO formation. Patients with chronic inflammation, therefore, suffer from Fe deficiency (hypoferremia) and high serum ferritin (hyperferritinemia), and thus develop anemia.

As noted above, bioavailable iron in mammals exists mainly within the hydrophobic methene-bridged tetrapyrrole ring of heme as a prosthetic group in hemoproteins. The most abundant of these are hemoglobin and myoglobin, with another significant pool of ubiquitously expressed hemoproteins formed by cytochromes. Mechanisms controlling host heme metabolism, including heme synthesis, transport (Yuan et al. 2013) and catabolism (Gozzelino and Soares 2014), play a critical role in the maintenance of host–microbe interactions as well as in the pathologic outcome of infectious diseases.

Iron homeostasis is also linked to Cu homeostasis through the Cu-based ferroxidases hephaestin (Figure 13.2) and ceruloplasmin. Thus, Cu deficiency prevents the incorporation of Fe^{3+} into ferritin and provokes an overload of free iron and Fe(hydr)oxide deposits. Comparable to the Fenton reaction (see above), Cu^{+} can promote the formation of reactive oxygen species (ROS). Cu/Zn superoxide dismutase (SOD) catalyzes the disproportionation (and thus detoxification) of superoxide to peroxide and dioxygen ($2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$). Thus, Cu deficiency prevents the effective removal of the ROS hyperoxide O_2^-. One of the many problems encountered with Zn deficiency points to the same direction. Similar considerations also apply for Mn-dependent SODs.

Impact on Anti-Immune Effector Functions

The development of anemia in inflammation and chronic disease not only limits the availability of iron for microbes, it also strengthens the immune response that is directed at invading pathogens. Specifically, Fe loading of monocytes/macrophages inhibits interferon gamma (IFN-γ)-mediated pathways, such as formation of TNF-α, reduced expression of the major histocompatibility complex class II antigens and the intracellular adhesion molecule 1, decreased formation of neopterin, and impaired tryptophan degradation via IFN-γ mediated induction of indole-amine-2,3-dioxygenase (Nairz et al. 2010). As a result, Fe-loaded macrophages have an impaired potential to kill various bacteria, parasites, and fungi (such as *Legionella*, *Listeria*, *Ehrlichia, Mycobacteria, Salmonella*, *Leishmania*, *Plasmodia*, *Candida, Mucor*) as well as viruses, *in vitro* and *in vivo* through IFN-γ mediated pathways (see also Cavet et al., this volume). Part of this can 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 (see above). Iron blocks the transcription of inducible NO synthase (iNOS or NOSII), the enzyme responsible for cytokine-inducible high-output formation of NO by hepatocytes or macrophages. This is attributed to a direct influence of iron on the binding affinities of transcription factors, such as the nuclear factor for the expression of IL-6 or the hypoxia inducible factor-1 (Nairz et al. 2010). According to the regulatory feedback loop, NO produced by activated macrophages activates the iron responsible element (IRE) of the binding function of the iron regulatory protein (IRP-1), leading to inhibition of ferritin translation, and thus linking maintenance of Fe homeostasis to NO formation for host defense. Through its deactivating effect on the IFN-γ function, iron also affects the balance of the thymus helper (Th) cells T_H1/T_H2. T_H1 effector functions are weakened, whereas T_H2-mediated cytokine production, such as the activity of IL-4, is increased, a condition which is rather unfavorable in an infection (Mencacci et al. 1997; Nairz et al. 2010). Iron overload also has negative effects on neutrophil function, as Fe therapy of chronic hemodialysis patients impairs the potential of neutrophils to kill bacteria, thus reducing their capacity to phagocyte

foreign particles (Weiss 2002). By modulating cytokine activities, iron also triggers macrophage polarization, and opposing 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 the heme oxygenase HO-1 has been linked to immune tolerance in infections, specifically malaria.

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

In one specific study, Fe-deficient children in Malawi featured exhibited a reduced incidence of infection, paralleled by (a) a higher percentage of CD8+ cells[1] which produced IL-6, (b) a more pronounced expression of lymphocytes with T cell activation markers, and (c) an increased formation of IFN-γ as compared to children with a normal Fe status (Oppenheimer 2001). This observation coincides with an *adverse* outcome in children receiving Fe supplementation, mainly as a consequence of an increased incidence of severe malaria and bacterial infection (Sazawal et al. 2006), and an *improved* outcome in children with cerebral malaria who received the Fe chelator desferrioxamine, stimulating the antimalarial immune responses. In Africa, an endemic form of secondary Fe overload—traced back to the consumption of traditional Fe-containing beer and linked to a mutation in the ferroportin gene (Gordeuk et al. 2003; Navarro and Visbal, this volume)—is associated with an increased incidence and mortality from tuberculosis. These data are supported by *in vitro* findings which show that changes in intramacrophage Fe availability stimulates the proliferation of mycobacteria and weakens antimycobacterial defense mechanisms of macrophages. Other infections, ranging from bacterial, viral and fungal to parasitic diseases, where Fe overload is associated with an unfavorable course of infection and/or an impaired immune response, have been reviewed (Weinberg 1999).

The importance of iron for 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 *Salmonella typhimurium* increase expression of ferroportin, which results in a stimulation of cellular Fe export and a limitation of Fe availability for intramacrophage bacteria, and leads to improved control of bacterial proliferation by macrophages. This is partly due to the fact that a reduction of cytoplasmic iron increases the activity of immune

[1] CD8 (CD stands for cluster of differentiation) is a transmembrane glycoprotein; CD8+ is short for cytotoxin T cells with a CD8 surface protein.

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 activates the transcription factor Nrf2 (a regulator of the antioxidant defense in macrophages), which 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 when mice are treated 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 by a number of intracellular bacteria, such as *Chlamydia* sp., *Legionella*, *Salmonella*, or *Mycobacteria* (Nairz et al. 2010).

Another immune gene that exemplifies the role of iron for infection is the phagolysosomal protein Nramp1 (natural resistance-associated macrophage protein 1). Expression of Nramp1 is associated with resistance toward infections by intracellular pathogens such as *Leishmania*, *Salmonella*, or *Mycobacteria* spp. mainly by shuttling divalent metals across the phagolysomal membrane (Blackwell et al. 2001; Forbes and Gros 2001). Investigations of the RAW264.7 macrophage cell line stably transfected with functional or nonfunctional Nramp1 demonstrated that macrophages which lack functional Nramp1 exhibited a significantly higher Fe uptake via the TfR and, as a consequence, an 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 to 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 reflected by increased formation of NO or TNF-α, whereas the 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 (also known as siderocalin or NGAL) (Fritsche et al. 2012). Lipocalin 2 is a neutrophil (gelatinase-associated) and macrophage-derived peptide which 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, lipocalin 2 delivers siderophore-bound iron to mammalian cells that are able to import the complex via a lipocalin 2 receptor. Most interestingly, recent data provide evidence for the existence of mammalian siderophores (Pantopoulos et al. 2012) that are captured by lipocalin 2, thus

indicating that lipocalin 2 may be involved in transcellular and transmembrane Fe trafficking in mammals.

In addition, lipocalin 2 expression affects neutrophil recruitment to the sites of infection which, depending on the underlying pathogen, exerts contrasting effects on infection outcomes (Warszawska et al. 2013). Interestingly, lipocalin 2 is also secreted during infection with non-siderophore-producing pathogens, such as *Chlamydia* or *Plasmodia*. Concomitantly, lipocalin limits Fe availability for *Plasmodia*, thereby impairing erythropoiesis. Impaired erythropoiesis, in turn, restrains the replication of *Plasmodia*. Thus, through the mechanisms described above, lipocalin 2 stimulates innate immune responses via limitation of Fe availability (Zhao et al. 2012). By a similar mechanism, lipocalin 2 may confer resistance to infection by the intracellular bacterial species *Salmonella* and *Mycobacteria* in patients suffering from hereditary hemochromatosis. As a consequence of reduced hepcidin formation and increased expression of lipocalin 2 upon infection, macrophages of these patients are Fe deficient and are thus 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. It has been shown that hepcidin expression negatively affects the proliferation of intrahepatic sporozoites, but may also affect the susceptibility to infection with Fe-dependent pathogens, such as *Salmonella* (Portugal et al. 2011). Accordingly, it appears that different regulatory mechanisms are initiated, depending on the type of the infectious pathogens. Hepcidin induction appears to be a very efficient strategy to limit Fe availability in extracellular bacteria, where nutrient iron is restricted within the reticulo-endothelial system. This strategy appears, however, to be detrimental for intracellular pathogens, where multiple pathways lead to the mobilization and export of iron out of macrophages, rendering them Fe deficient—a fact that ameliorates the control of infection with intracellular microbes and also positively affects innate immune responses.

Copper

Copper is present in a variety of essential redox enzymes such as cytochrome oxidase, Cu/Zn-SOD, and others. Importantly, copper is also a critical cofactor for high-affinity Fe uptake in fungi, Fe efflux from intestinal epithelial cells and macrophages, and Fe loading onto transferrin in mammals (see Figure 13.2 for the interplay of iron and copper).

In mammals, copper is absorbed at the apical surface of the intestinal epithelium primarily by the high-affinity Cu(I) importer Ctr1 in a manner involving cell surface Cu(II) metalloreductase activity. Copper uptake at the apical surface is modulated by Cu-dependent endocytosis of Ctr1, but as high Zn levels can block intestinal Cu absorption, additional Cu transport mechanisms (at the cell surface or at the basolateral membrane) may be involved. Within

the cytosol, copper is distributed to enzymes, such as Cu/Zn-SOD, or to the secretory compartment by Cu chaperone proteins CCS (Cu chaperone for SOD) and Atox1 (antioxidant protein 1), respectively. Atox1 transfers copper to the Cu-transporting P-type ATPases, ATP7a, expressed in the intestine, and ATP7b, expressed in the liver and other tissues, for import into the lumen of the trans-Golgi network for loading onto secreted Cu-dependent proteins. Under elevated Cu concentrations, intestinal epithelial ATP7a localizes to the basolateral membrane, where it mobilizes copper into the circulation; consequently, mutations in the *ATP7a* gene cause Menkes disease, an X-linked peripheral Cu deficiency that typically results in death by 2 or 3 years of age. In contrast, mutations in the *ATP7b* gene result in hepatic Cu accumulation: normally, ATP7b excretes biliary Cu, and neuronal dysfunction results from Cu overload.

Copper is clearly required for innate immune cell function in at least two ways. First, copper drives the development of neutrophil myeloid progenitor cells. As the mechanisms for this are as yet unknown, understanding the role of copper in neutrophil development represents a key area for further investigation. As dietary Cu deficiency or cancer chemotherapy lead to neutropenia and predispose mammals to infectious diseases, knowledge in this area is needed to determine dietary or genetic predisposition to infection via inadequate Cu homeostasis or dysfunction of the Cu homeostatic machinery. This is particularly relevant since neutropenia is one of the most sensitive consequences of dietary Cu deficiency. Second, recent accumulating evidence supports a microbiocidal role for copper in the macrophage phagosomal lumen. In response to proinflammatory conditions, expression of both Ctr1 at the plasma membrane and ATP7a (which partially localizes to the phagosomal membrane) is elevated, driving Cu accumulation in the lumen, as ascertained by X-ray fluorescence microscopy studies. RNA iterference-mediated knock down of ATP7a compromises macrophage antibacterial activity, providing strong evidence for the importance of Cu compartmentalization in the phagosome as one of several important host defense mechanisms. Still, the mode by which phagosomal copper is particularly potent in killing microbial pathogens is not well understood. *Mycobacterium tuberculosis* has three independent Cu-resistance pathways that involve the Cu-sensitive operon repressor (CsoR), the mycobacterial Cu transport protein B (MctB), and the regulated-in Cu repressor (RicR) (Shi et al. 2014). Because *M. tuberculosis* is a human-exclusive pathogen, the evolution of this bacterium to possess three Cu-resistance pathways strongly suggests that it encounters toxic levels of Cu in the host and that this may be driving multiple pathways for resistance.

There are varying reports on increased incidence of microbial infection in patients suffering from Menkes disease. However, the precise impact of Cu deficiency on this observation may be obfuscated by the plethora of clinical phenotypes presented in patients that are both a direct or indirect consequence of peripheral Cu deficiency. Future studies will need to elucidate mammalian

sources of antimicrobial copper; in particular, ceruloplasmin, an abundant multi-copper oxidase which may play a role in addition to (or perhaps in some instances in place of) ATP7a. Moreover, mice supplemented with copper are better able to control tuberculosis growth (Rowland and Niederweis 2013). In mice, the RicR regulon is required for Cu resistance and virulence (Shi et al. 2014). Copper-resistance mechanisms, via the Cu-specific activation of metallothionein gene transcription, also play a role in *Cryptococcus neoformans* (a fungus) infections (Ding et al. 2013).

Zinc

Distribution during Infection

During bacterial infection zinc rapidly shifts from the serum into the liver (Rink 2011). Zinc uptake and intracellular distribution is mediated by Zn transporters from two families: Zn transporter (ZnT, SLC30A1-10) and Zrt- and Irt-like proteins (ZIP, SLC39A1-14). ZIPs increase cytosolic zinc whereas ZnTs decrease it. Knowledge about the concentration of tissue zinc is incomplete. Serum zinc drops down, since the Zn transporter ZIP14 is upregulated in the liver (Sayadi et al. 2013); this results in tissue Zn accumulation. ZIP14 expression is induced by the proinflammatory cytokines IL-6 and IL-1 (Lichten and Cousins 2009). In other infectious diseases, this shift of zinc has not been directly shown, but since the effect is mediated by cytokines also released during parasitic, viral, and fungal infections, this seems to be a general effect.

On the cellular level, Zn redistribution is more complicated. Some studies have shown that, in macrophages, zinc is accumulated in the cytoplasm, whereas it is depleted in the phagolysosomes. Enrichment of zinc in the phagolysosomes has also been noted. A detailed comparison of the studies revealed that zinc is enriched during bacterial infections but is depleted during infections with protozoa (Haase and Rink 2014). This may imply that the macrophages are able to distinguish between different types of pathogens, and thus choose the appropriate defense mechanism. Since zinc in high concentration is toxic for bacteria (Haase 2013), this would be the obvious approach for this type of infection, whereas Zn deficiency could induce programmed cell death in eukaryotic cells.

Role in Host Resistance, Susceptibility, and Tolerance

The essentiality of zinc for the immune system has been known for decades. Zinc deficiency always results in an immune deficiency (Rink 2011). Generally, one has to distinguish between a general effect of zinc on the cell cycle and proliferation, and specific effects toward immune cells. Since the immune system is the organ system with the highest proliferation rate in the human body (e.g., 80 million neutrophils are released from the bone marrow per minute),

any micronutrient deficiency with an influence on cell proliferation will reduce the immune response due to leukopenia.

However, several specific effects of zinc on the immune system are known (Rink 2011). The first observation revealed a reduction of the number of T cells due to thymus atrophy. Atrophy of the thymus is induced by a lack in active thymulin, a nonapeptide thymic hormone that is only active in its Zn-bound state (Figure 13.4). Thymulin is involved in T cell differentiation. The influence of Zn deficiency on the B lymphocyte system is less pronounced. There is no effect on mature B cells, whereas the number of B cell precursors is reduced. The number of monocytes/macrophages and dendritic cells is increased during Zn deficiency, since Zn deficiency promotes the differentiation of myeloid cells into monocytes/macrophages as well as dendritic cell maturation. Differentiation of neutrophils, however, is not influenced by Zn deficiency (Haase and Rink 2014).

Furthermore, Zn deficiency results in a proinflammatory phenotype of the immune system, depending on the increased number of macrophages as well as on direct epigenetic effects on proinflammatory cytokine genes (Wessels et al. 2013). Zinc-deficient macrophages produce the proinflammatory cytokines, the interleukins IL-1, IL-6, and TNF-α without a danger signal, normally needed for the induction. At least for IL-1, zinc opens the promoter of the IL-1 gene, leading to an enhanced transcription of the gene (Wessels et al. 2013). Interestingly, the promoter is closed after addition of exogenous zinc, so that the activity of the gene is directly regulated by the cellular Zn concentration.

While the monocytes/macrophages show a chronic inflammatory status due to Zn deficiency, the T helper cell (T_H1) system exhibits a reduced capacity, resulting in a decreased production of IL-2 and IFN-γ. These two effects trigger chronic inflammatory diseases and an increased number of infections (Rink

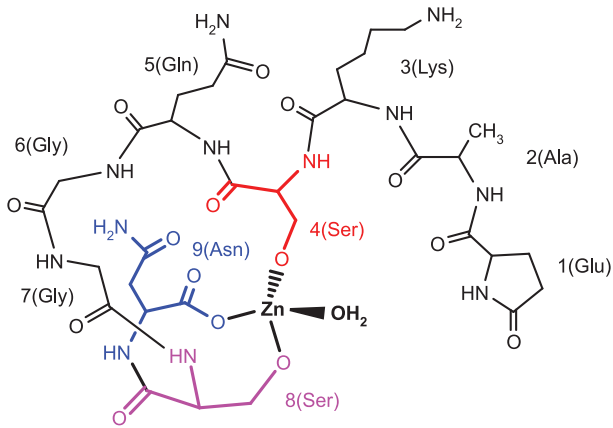

Figure 13.4 Binding of Zn^{2+} to thymulin. The zinc ion is in a tetrahedral environment, coordinated to a water molecule and side-chain oxygen functions of two serinates (red and mauve) and one aspartate (blue) of the nonapeptide (after Dardenne and Pleau 1994).

2011). In contrast to the T_H1 system, the T_H2 system remains almost uninfluenced by Zn deficiency, in regard to cytokine production. However, the signal transduction of IL-4 is diminished in the case of Zn deficiency (Rink 2011; Gruber et al. 2013). Combined, both effects result in T_H2 dominance during Zn deficiency. Zinc supplementation restores the T_H1 system, inducing a normal production of IL-2 and IFN-γ.

Cellular activation is also influenced by Zn deficiency, since the release of zinc into the cytoplasm is a signal comparable to that otherwise initiated by calcium (Haase and Rink 2009). Zinc signals are generated by TLRs, the T cell receptor, and some cytokine receptors (Haase and Rink 2009, 2014; Rink and Maywald 2014). Therefore, Zn deficiency results in a lack of an appropriate signal of the respective receptor. The function of zinc in the signaling process is not completely understood, but one effect is the inhibition of phosphatases stabilizing the signal induced by kinase activities. Clearly described examples are the mitogen-activated protein kinase phosphatase and PTEN phosphatase and tensin homolog in IL-2 signaling (Haase and Rink 2014). However, signal transducer and activator of transcription STAT-5 phosphorylation in IL-2 signaling is not influenced, showing that zinc has some specificity in its inhibitory capacity. On the other hand, STAT-6 phosphorylation induced by IL-4 is negatively influenced by Zn deficiency, whereas STAT-3 phosphorylation induced by IL-6 is increased (Gruber et al. 2013). A complete picture of the Zn-regulated signaling pathways is still missing, but a summary of the most important pathways is illustrated in Figure 13.5.

Finally, zinc is important for the induction of regulatory T cells (Treg) and for tolerance induction. Zinc stabilizes the Treg-specific transcription factor Foxp3 due to an inhibition of the histondeacetylase sirtuin-1 (Haase and Rink 2014; Rink and Maywald 2014). Normally, sirtuin-1 deacetylates Foxp3 resulting in an ubiquitinylation and degradation of this transcription factor. When sirtuin-1 is inhibited, Foxp3 will not be degraded, eventuating in a regulatory phenotype of the T cells. Therefore, Zn homeostasis is important to maintain the complete capability of the immune system to interact with immune activation or tolerance. Thus, Zn deficiency is not only accompanied by a high frequency of infections, but also by an increase of allergies and autoimmune diseases (Rink 2011).

Excess zinc also exerts negative effects on the immune response, although this is not as stringently defined as Zn deficiency. Whereas slightly increased Zn levels inhibit phosphatases only, unphysiologically high concentrations have been shown to inhibit kinases, thereby interrupting signaling by various receptors. Furthermore, high Zn concentrations decrease the fluidity of membranes and thereby reduce the clustering of receptors normally needed for an effective signal transduction. High Zn concentrations also have negative effects on differentiation and maturation of monocytes/macrophages (Haase and Rink 2009; Rink 2011).

Therapeutic Effects in Infectious Diseases of Children

The recognition of the many roles of zinc in human immune and nonimmune defenses against infection, and the increased rates of infectious diseases with severe Zn deficiency syndromes have led to a hypothesis that children with milder degrees of Zn deficiency may be at increased risk of infections. If true, this could result in a large disease burden due to the high prevalence of Zn deficiency and high rates of infectious diseases in low- and middle-income countries. Because of the frequent co-occurrence of Zn deficiency with other nutritional deficiencies, the limitations of determining the Zn status in individuals, and the potential confounding of the relationship with socioeconomic conditions affecting the Zn status and infections, observational studies are not suited to test this hypothesis. Thus, numerous randomized controlled trials, in which the effects of zinc can be segregated, have been conducted. Some of these trials have been focused on the treatment of specific childhood infectious diseases, while others have sought to determine whether daily or weekly Zn supplementation can reduce the incidence of infectious diseases. In all trials where this hypothesis has been assessed, children receiving zinc versus those not receiving zinc is the sole variable between the comparison groups.

Most trials have been done to assess the effect of zinc in treating diarrhea in children. The first definitive trial in India found a 23% (95% Confidence Interval 12–32%) reduction in diarrhea duration (Sazawal et al. 1995). In the nearly two decades since that trial was published, more than a hundred randomized trials of zinc in treatment of diarrhea have been conducted. Overall they confirm that Zn supplementation reduces the diarrhea episode duration by about one quarter (Lamberti et al. 2013). Furthermore, trials done in diarrhea due to specific etiologies have shown a similar effect in childhood illness caused by *Shigella* sp. and *Vibrio cholera*. Clinical trials in health facilities and community-based trials of Zn supplementation for diarrhea treatment have shown that this has some preventive effects also for diarrhea and lower respiratory diseases; the community studies further show a reduction in hospitalizations from diarrhea and pneumonia, and in child mortality (Baqui et al. 2002). These findings resulted in a recommendation, in 2004 from WHO and UNICEF, that zinc (20 mg per day) be used along with fluids to manage all childhood diarrhea. Some, but not all, trials of zinc in the treatment of pneumonia have found benefit, and a recent trial in young infants with probable serious bacterial infection showed a reduction in treatment failure rates with Zn supplementation.

Trials to evaluate the possible preventive effect of daily or weekly Zn supplementation have also been widely done. A recent meta-analysis included 33 comparisons involving almost 17,000 children (Brown et al. 2009). Overall there was a 20% (95% Confidence Interval 10–29%) lower incidence of diarrhea in children who received zinc. A preventive effect of zinc on acute lower respiratory infections has also been demonstrated. Very large randomized

controlled trials of Zn supplementation have been carried out in Zanzibar and Nepal (Sazawal et al. 2007; Tielsch et al. 2007). These showed a reduction of 18% (95% Confidence Interval 4–30%) in total mortality in children 12–47 months of age.

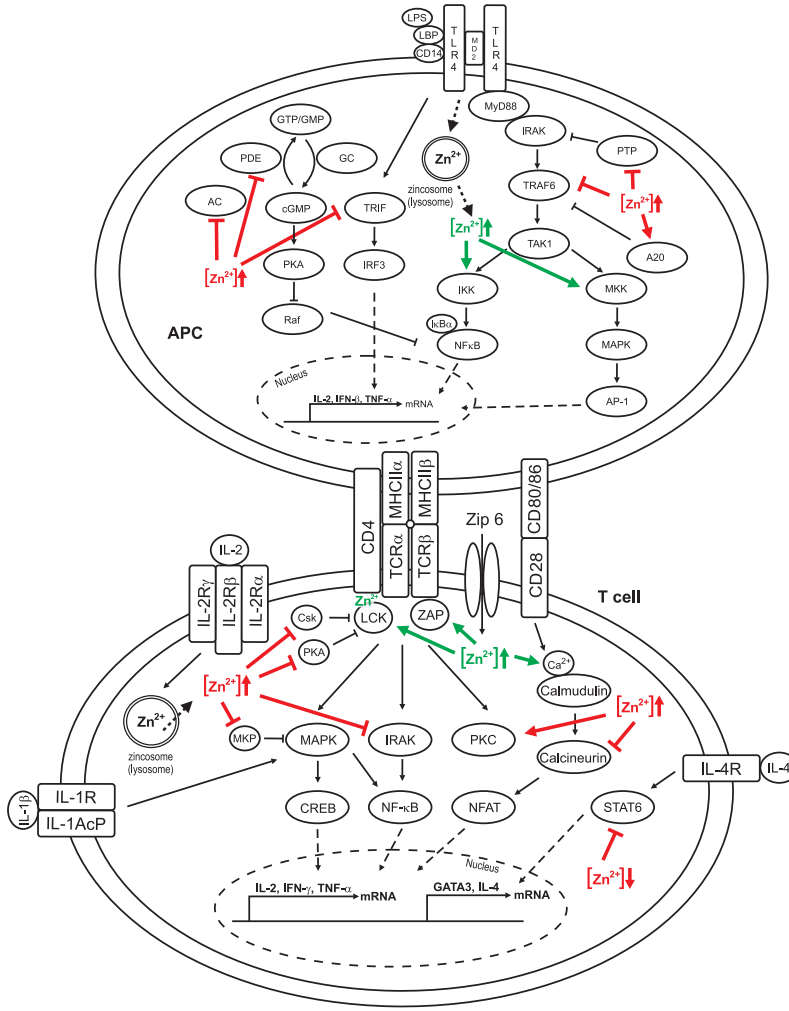

Figure 13.5 Influence of zinc on the signaling pathways (modified after Rink and Maywald 2014). Top: Zinc interacts with a multitude of signaling pathways in different leukocyte subsets. During the activation of the adaptive immune system, the central interaction (depicted) takes place between antigen-presenting cells (APC) and T cells. Zinc, however, influences the same (as well as some other) pathways in different leukocyte subsets. APCs are activated by pattern recognition receptors, e.g., Toll-like receptors (TLRs). All TLRs, with the exception of TLR-3, generate a Zn signal after stimulation with their specific ligand.

Figure 13.5 (continued) For example, the activation of TLR-4 by lipopolysaccharide (LPS) from Gram-negative bacteria is shown to induce a fast Zn release (dotted arrows) from zincosomes. This fast Zn signal (in green) is physiologically required for nuclear factor kappa B (NFκB) activation (solid arrows) as well as for the mitogen-activated protein kinase (MAPK) signaling, both resulting in translocation of the appropriate transcription factors into the nucleus (dashed arrows). However, increasing Zn concentration for a time (e.g., after use of ionophores, highlighted in red) inhibits even TLR-4 signaling (⟞). This effect appears to be mediated by direct inhibition of the IL-1 receptor-associated kinase (IRAK), a de-ubiquitination of the tumor necrosis factor receptor-associated factor 6 (TRAF-6) by upregulation of A20, a cyclic nucleotide phosphodiesterase (PDE) inhibition or an inhibition of TIR-domain-containing adapter-inducing interferon-β (TRIF). PDE inhibition results in an increase of cyclic guanosine monophosphate (cGMP). cGMP activates protein kinase A which inhibits RAF directly and NFκB indirectly. Lastly, increased intracellular Zn concentrations persisting for a longer time have the opposite effect due to an inhibition of adenylate cyclase (AC) transcription.

Bottom: On the T cell side, zinc influences the signaling of the T cell receptor (TCR), as well as the signaling pathways of at least IL-1, IL-2, and IL-4. A Zn signal is directly induced by ZIP 6 due to an APC-mediated activation (green) of TCR. This results in augmented ZAP phosphorylation, sustained Ca^{2+} influx, and downstream TCR signaling by binding of the lymphocyte-specific protein tyrosine kinase (LCK). The binding of IL-2 to its receptor (IL-2R) induces a Zn release from zincosomes (dotted arrow). The increased intracellular Zn concentration (red) mediates IRAK inhibition (⟞), as described for APCs, and mediates c-Src tyrosine kinase (Csk)/protein kinase A (PKA) inhibition of LCK in TCR signaling. It also induces (solid arrows) a zeta-chain (TCR)-associated protein kinase (ZAP), and protein kinase C (PKC) activity, MAPK signaling, and NF-κB phosphorylation. Lastly, calcineurin (CN) is inhibited by increased intracellular Zn concentrations, avoiding translocation into the nucleus (dashed arrows) of the nuclear factor of activated T cells (NFAT). However, Zn deficiency also influences cytokine signaling in T cells. As an example, phosphorylation of the signal transducer and activator of transcription 6 (STAT6) in IL-4R signaling is decreased during Zn deficiency.Additional abbreviations: IKK, I kappa B kinase; IRF3, interferon regulatory factor 3; MKK, MAPK kinase; PKA, protein kinase A; PTP, protein tyrosine phosphatases; AP-1 activator protein 1; GMP, guanosine monophosphate; GTP, guanosine triphosphate; MKP, MAP-kinase phosphatase; CREB, cyclic adenosine monophosphate response element-binding protein.

Open Questions

In this discussion on zinc, ambiguities with respect to Zn homeostasis, the mode of operation of zinc, and Zn supplementation in the case of infectious diseases have been insinuated. To some degree, these open questions also apply to other essential metals. Thus, we briefly summarize core issues that await resolution:

- Is zinc needed for liver metabolism (e.g., in acute phase reaction)?
- Is transient hypozincemia an activation signal for the immune system?
- Does hypozincemia (the withholding of zinc from bacteria) constitute a similar defense mechanism as in iron?
- How does Zn metabolism interact with the metabolisms of iron, copper, and manganese?

- What are the actual molecular targets of zinc within the cell and/or in specific cell types? There are 14 Zn importers (ZIPs, responsible for an *in*crease of cytoplasmatic zinc) and 10 Zn transporters (ZnTs, responsible for a *de*crease of cytoplasmatic zinc); however, their locations (cell type, compartment) are not completely understood yet.
- How are parasites and microbes affected by high or low Zn levels, and what are the mechanisms of the therapeutic effects of zinc?
- How are compartmentalization and speciation of zinc related?
- How do toxic metal ions (Pb^{2+}, Hg^{2+}, Cd^{2+}) "compete" with zinc and interact with Zn-containing enzymes and storage proteins (thioneins)?

Selenium

Background

Selenium is incorporated into a variety of selenoproteins whose functions (e.g., calcium flux, oxidative burst, redox signaling, and effector immune functions) can exert a dramatic impact on inflammation, immunity, and pathogen status (Huang et al. 2012b). Selenium was among the first micronutrients to be characterized in several species for alteration of immune function, including its immunological relationship to other antioxidant-modulating dietary factors (such as vitamin E). In humans, lower Se plasma concentrations have been associated with intensive care unit patients who experience severe infections, increased tissue inflammation, subsequent organ dysfunction, and mortality (Sakr et al. 2007). This general relationship is supported by numerous animal studies, which link Se status to innate immune defenses against pathogens in affected tissues, virulence of certain pathogens, and the risk of immune-mediated organ damage.

Susceptibility to Viral and Bacterial Infections and to Parasites

Evidence suggests that Se status and, in particular, Se deficiency can affect the host–pathogen interaction in viral infections. In mouse models, mildly virulent forms of two different viruses, coxsackievirus B3 and influenza, underwent unexpected genetic conversion to highly virulent forms with elevated risk of specific host pathology. The status of the antioxidant selenoenzyme glutathione peroxidase apparently plays a role in this host–pathogen alteration. Examples of similar nutritional–viral pathogenesis interactions appear to occur in humans (Beck et al. 2003).

Evidence suggests that both Se deficiency and supplementation can affect host immunity and resistance to bacterial infections. Selenium deficiency appears to target innate immune cell function, resulting in increased susceptibility to several bacterial diseases. With Se-deficient mice, innate immune-mediated protection was reduced and burdens of *Listeria monocytogenes* were

elevated in several tissues over the course of the infection compared with dietary controls (Wang et al. 2009). Selenium supplementation has been reported to protect against chronic bacterial (*E. coli*) prostatitis in a rat model. Alone, selenium reduced bacterial infection and lowered inflammatory cell infiltration of the prostate. In addition, Se supplementation enhanced the effectiveness of antibiotic treatment (Kim et al. 2012).

Selenium also exerts direct effects on bacteria. Organoselenium compounds have been reported to inhibit the formation of biofilms among bacteria such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Tran et al. 2009). This has implications for both medical and dental procedures, where Se-based coatings have been applied to polycarbonate medical devices as well as in dental sealants.

Selenium status affects both the host immune response and parasite metabolism. In the nematode infection of *Heligmosomoides bakeri* in mice, adequate Se supplementation affects local tissue T_H2 response, resulting in worm expulsion. With Se deficiency, the T_H2-driven immune response in tissues is inadequate, glutathione protection is reduced, and the adult worms increase their metabolism, leading to enhanced pathogen success. In this model, dietary restoration of Se levels can rapidly result in an effective host defense and worm expulsion (Smith et al. 2013). Selenium supplementation appears to produce host resistance benefits against trypanosomes. In rats infected with *Trypanosoma bruceri*, dietary Se supplementation beginning two weeks prior to infection resulted in reduced anemia and parasitemias and increased survival intervals (Eze et al. 2013).

Evidence suggests that combined Se and vitamin E deficiency can exert a significant effect on the host immune status and pathogen susceptibility well beyond that of either single deficiency. With *Citrobacter rodentium* infection in mice, double-deficient animals had increased immune cell infiltration of the colon with elevated production of both proinflammatory cytokines and markers of oxidative stress. Bacterial burden was significantly elevated in the double-deficient animals (Smith et al. 2011).

Recommended Dietary Allowances and Related Intake Levels

The actual guideline for nutritional metal intake for healthy people is the RDA, differentiated according to sex, age, pregnancy, and lactation. Values vary for different countries (United States, China, European Union, Australia). Daily RDAs range between 3–22 mg for Zn, 6–15 mg for Fe, 0.2–1.7 mg for Cu, and 15–200 µg for Se. These guidelines are a source of information for nutritional advice and attempt to reduce the risk of diseases, including infectious diseases related to metal deficiency. For zinc, the missing differentiation in RDAs between the age groups 14–50/70 and >70 years of age comes as a surprise, since

the bio-recovery of zinc decreases with age (Chasapis et al. 2012). For iron, the RDA for males appears to be rather high, since just 1–2 mg are excreted daily.

Along with RDAs come recommendations which list tolerable upper intake levels (UIL). The UIL defines the highest average daily intake that is likely to pose no risk for adverse health effects, and hence is a tolerable uptake level; however, they are *not* recommended as being *beneficial* to an individual. In particular, the UIL should be considered (and even challenged) in the case of self-medication (e.g., nutritional supplements available in drugstores and supermarkets). Daily UILs range between 10–50 mg for iron, 4–40 mg for zinc, 1–10 mg for copper and 45–450 μg for selenium.

For a detailed overview and corresponding tables of RDAs and UILs in relation to age, sex, and geographical areas, see Wang and Zhang (this volume).

RDAs and UILs do not directly reflect the bioavailability of the metals; that is, to what extent metal ions present in nutrients (and supplements) can actually be disposed of and utilized by an individual. As far as artificial formulations are concerned, absolute and relative bioavailability are distinguished: absolute bioavailability compares bioavailability following nonintravenous versus intravenous application; relative bioavailability compares bioavailability from two differing formulations applied intravenously. Further, bioavailability depends on whether a metal is applied in the form of an inorganic compound, a metal ion coordinated to an organic chelate (e.g., an amino acid), or a conjugate between yeast and an inorganic salt, etc.

In addition, the bioavailability of metals depends on factors associated with characteristics of the respective individual (e.g., age, sex, health, food situation, gut flora). Bioaccumulation and biomagnification of metals can cause nutritional overloads of essential metals/metalloids that are required only in minor amounts, such as copper, molybdenum, and selenium. Furthermore, interaction between different metals can influence their uptake and distribution (e.g., by transporters for divalent metal ions), as discussed above.

Metal-Based Drugs in the Treatment of Tropical Parasitic Diseases

Metal-based drugs are used therapeutically to treat cancer (cisplatin, carboplatin, and oxaliplatin), arthritis (the gold compounds solganol, myocrisin, and auranofin), and parasitic infections caused by leishmaniasis and schistosomiasis (antimony compounds based an antimonite plus gluconates) (see Navarro and Visbal, this volume). Examples of platinum, gold, and antimony complexes used medicinally are shown in Figure 13.6. In addition, silver and silver compounds are used as disinfectants, and other compounds, such as antidiabetic bis(maltolato) oxidovanadium(IV), have passed phase II clinical tests. Metal-based drugs are otherwise not in common use, and it does not appear that pharmaceutical companies commonly have interest in drugs that contain a metal core.

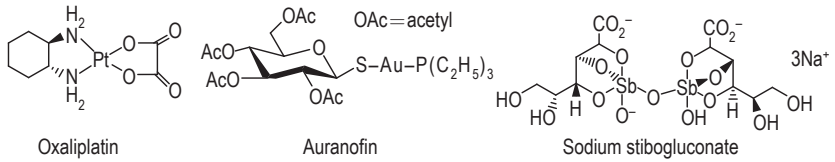

Figure 13.6 Examples for coordination compounds of metals that are in medicinal use: oxaliplatin (colorectal cancer), auranofin (rheumatoid arthritis), and sodium stibogluconate (leishmaniasis).

Earlier we addressed the benefits that Se supplementation brings against trypanosomes, organisms which cause sleeping disease and Chagas disease (see also Navarro and Visbal, this volume). Promising *in vivo* and *in vitro* studies have used diverse metal complexes and shown that they are just as effective as antiparasitic drugs in combating leishmaniasis, Chagas disease (*American trypanosomiasis*), amoebiasis, and malaria. These drugs exploit the metal–drug synergism: targeting is more efficient (with respect to the employment of just the organic constituent), and drug stabilization equates to longer residence time. Thus, coordination compounds of ruthenium, copper, rhodium, platinum, and gold (Navarro et al. 2010; Biot et al. 2012), as well as vanadium (an early transition metal; for a review, see Rehder 2012) have successfully been tested for their antimicrobial activity against *Trypanosoma* and *Leishmania* parasites. Examples of efficient ligand systems are hydrazones, semicarbazones, fluorodiketones, sulfonamides, and 8-aminoquinoline. Coordination complexes of copper, silver, ruthenium, and platinum, which contain aromatic or pseudo-aromatic ligands for the potential intercalation in DNA, have shown promising *in vitro* activity against *Trypanosoma cruzi* and *Leishmania major* promastigotes. In many cases, the ligands in this group of complexes derive from ortho-phenanthroline (Figure 13.7), and the action of these complexes suggests a mechanism comparable to that of anticancer drugs.

The increasing resistance of malaria parasites against chloroquine—the classical remedy for malaria infections—has spurred the development of metal (Fe, Au, Ru, and Pt) complexes based on chloroquine and its derivatives. These complexes circumvent resistance against chloroquine by preventing the conversion of hematin to the stable dimer hemozoin:

$$\underset{Fe^{2+}}{\text{heme}} \xrightarrow{\text{oxidation}} \underset{Fe^{3+}(OH)\text{–heme}}{\text{hematin}} \xrightarrow{\text{“dimerization”}} \text{hemozoin} \qquad (13.1)$$

Coupling between two hematins takes place through the formation of coordinative bonds between Fe^{3+} and carboxylate. Hematin, i.e., Fe^{3+}(OH)-heme, is generated through the oxidation of heme (containing ferrous iron), which is toxic for the malaria parasite. In addition, ferrocenyl-derivatized amino-chloroquinoline has been shown to function according to a comparable mechanism. For additional details and references, see Navarro and Visbal (this volume)

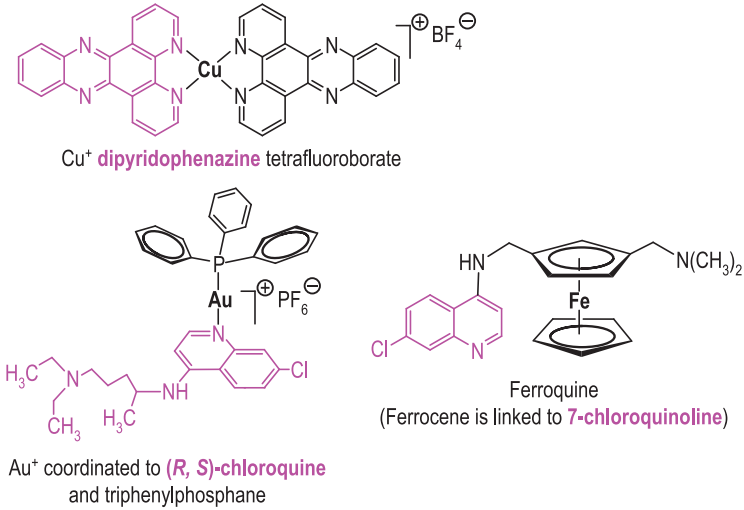

Figure 13.7 Three coordination compounds with promising potential in the treatment of tropical diseases: the copper complex with the dipyridophenazin ligand system is active against trypanosomiasis, whereas the gold complex (based on the chloroquine ligand) and ferroquine are active against malaria parasites.

and recent reviews on the development of metallopharmaceuticals to combat malaria (Navarro et al. 2012; Salas et al. 2013).

In conclusion, diverse (transition) metal ions can be used to treat parasitic tropical diseases. The metal commonly functions by stabilizing the active component (the specific ligand in the coordination compound) and by enhancing the drug's activity in the sense that the metal ion improves the drug's lifetime, its target specifity, and the efficacy of binding into the target site. A metal-based drug can also function through the delivery of metal ions to a specific site of action, in particular because the metal switches easily between different oxidation states, such as $Fe^{2+/3+}$ and $Au^{+/3+}$.