Immunological Reviews

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Mycobacteria, metals, and the macrophage

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Acknowledgements
We thank Jennifer Rowland for the figure design and Alex Dalecki and Kaitlyn Schaaf for editing the manuscript. Research in the laboratory of O.N. is supported by the Centre National de la Recherche Scientifique (CNRS), the Fondation pour la Recherche Médicale (FRM), the Agence Nationale de la Recherche (ANR), the European Union, the Fondation Mérieux, and the Bettencourt–Schueller Foundation. This work was funded by the National Institutes of Health grants AI104952 to F.W., and AI074805 and AI083632 to M.N. The authors have no conflicts of interest to declare.

This article is part of a series of reviews covering Tuberculosis appearing in Volume 264 of *Immunological Reviews*.

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Immunological Reviews 2015
Vol. 264: 249–263

Immunological Reviews
0105-2896

Summary: *Mycobacterium tuberculosis* is a facultative intracellular pathogen that thrives inside host macrophages. A key trait of *M. tuberculosis* is to exploit and manipulate metal cation trafficking inside infected macrophages to ensure survival and replication inside the phagosome. Here, we describe the recent fascinating discoveries that the mammalian immune system responds to infections with *M. tuberculosis* by overloading the phagosome with copper and zinc, two metals which are essential nutrients in small quantities but are toxic in excess. *M. tuberculosis* has developed multi-faceted resistance mechanisms to protect itself from metal toxicity including control of uptake, sequestration inside the cell, oxidation, and efflux. The host response to infections combines this metal poisoning strategy with nutritional immunity mechanisms that deprive *M. tuberculosis* from metals such as iron and manganese to prevent bacterial replication. Both immune mechanisms rely on the translocation of metal transporter proteins to the phagosomal membrane during the maturation process of the phagosome. This review summarizes these recent findings and discusses how metal-targeted approaches might complement existing TB chemotherapeutic regimens with novel anti-infective therapies.

Keywords: *phagosome, iron, copper, zinc, manganese, nutritional immunity, poisoning, innate immunity*

Introduction

Mycobacterium tuberculosis is a facultative intracellular pathogen that thrives inside host macrophages and other cell types, in which it resides in a membrane-bound vacuole, the phagosome, and can also escape into the cytosol at late stages of infection (1–3). The ability of *M. tuberculosis* to resist killing by macrophages relies mostly on its ability to arrest phagosome maturation, i.e. to manipulate the host cell endocytic machinery in order to prevent phagosome fusion with late endosomes and lysosomes (4, 5). Intracellular survival and replication of the bacillus also relies on the acquisition of various host compounds such as lipids and amino acids as carbon (6–8) and nitrogen (9, 10) sources. In addition, *M. tuberculosis* is well equipped to resist acid stress and reactive oxygen and nitrogen species that are copiously produced during infection (11).

A key trait of *M. tuberculosis* is to exploit and manipulate metal cation trafficking inside infected macrophages. Essential micronutrients, e.g. iron and manganese, are kept away from intracellular *M. tuberculosis* through sequestration by host proteins such as transferrin and ferritin or through efflux from the phagosome by the divalent metal cation transporter natural resistance-associated macrophage protein 1 (Nramp1) (12–14). To overcome iron deprivation, *M. tuberculosis* has evolved efficient iron capture systems based on the siderophores mycobactins (MBT) and carboxymycobactins (cMBT) (15, 16) and the recently identified capability of *M. tuberculosis* to utilize heme (17, 18). More recently, other metal cations, namely copper and zinc ions, were shown to accumulate inside the mycobacterial vacuole to toxic levels (19, 20). To resist metal intoxication, *M. tuberculosis* uses metal efflux and detoxification systems, such as P-type ATPases, oxidases and sequestration (21–23). In this review, we highlight the recent progress in metal biology of *M. tuberculosis* and the dual roles of several metals in host-pathogen interactions as micronutrients for the bacteria and toxic weapons for the host. In particular, we discuss the emerging concept that the host immune system has exploited this vulnerability by overloading *M. tuberculosis* with excess metals to kill the bacteria. Thus, the mammalian immune system in response to *M. tuberculosis* infection seems to combine nutritional immunity mechanisms by depriving *M. tuberculosis* from some metals (Fe, Mn), while poisoning the bacteria with others (Cu, Zn). These fascinating developments open novel venues to better understand host-pathogen interactions and to design new intervention strategies in tuberculosis therapy.

Metal acquisition by *M. tuberculosis* and its role in intracellular survival: the case of iron

The physiological role of iron

Iron switches readily between its two most prevalent oxidation states, Fe(II) (ferrous) and Fe(III) (ferric), and is therefore particularly suited to carry out single electron transfer reactions (24). Iron ions in both oxidation states form complexes with several ligands and different coordination numbers and geometries. This versatility enables fine-tuning of the redox potential of Fe(III)/Fe(II) between −500 mV to 600 mV in proteins and makes iron an ideal co-factor in many redox reactions including respiration and DNA synthesis (25). Not surprisingly, iron is an essential metal for all known bacterial pathogens with the notable exception of *Borrelia* spp. (26). Iron is abundant in the human body (27), but it is also one of the least accessible micronutrients due to sequestration by host proteins (28). Approximately 70–75% of the iron in the human body is bound to porphyrin to form heme, which is essential for oxygen transport, enzymatic reactions, and cellular respiration (24). As free heme is toxic due to its association with membranes, ~95% of host heme is bound by proteins (29). Iron that is not bound by heme is sequestered by the transport proteins transferrin and lactoferrin or stored in ferritin (30, 31). These host mechanisms usually keep free iron below the level required for bacterial growth and are regulated by the hormone hepcidin which orchestrates an innate immune response to further reduce available iron and to slow or stop growth of bacterial pathogens (32, 33).

However, iron can also be toxic, because it can generate highly toxic hydroxyl radicals from hydrogen peroxide (34), an endogenous byproduct of aerobic respiration (35). Although hydroxyl radicals react with most biomolecules, the damage inflicted on genomic DNA was considered for a long time as the principal mechanism accounting for the toxicity of hydroxyl radicals and thus of iron (34).

Iron acquisition by bacterial pathogens

To counter iron deficiency, bacterial pathogens have developed high affinity acquisition systems for iron-loaded siderophores, heme and for the host proteins transferrin and lactoferrin. Most bacteria secrete small iron chelators called siderophores, which bind ferric iron with high affinity and transport it into the bacterial cell (28, 36, 37). Iron utilization by *Staphylococcus aureus* is well studied and serves as a paradigm for Gram-positive bacteria (38). Binding of the iron-loaded siderophores staphyloferrin A and B by the lipoproteins HtsA and SirA, respectively, induces conformational changes leading to uptake by their cognate membrane-spanning permeases HtsBC and SirBC, respectively (39–42). The presence of a second membrane makes iron uptake by Gram-negative bacteria substantially more complicated. In *Escherichia coli*, iron-loaded siderophores, such as enterobactin and ferrichrome, are first bound by the outer membrane receptors FepA and FhuA, respectively, which transport the iron-loaded siderophores into the periplasm. The energy for this transport is derived from the electrochemical gradients across the inner membrane and is transduced by the TonB-ExbB-ExbD protein complex to the outer membrane receptors (43–45). Then, the iron-siderophore complex is bound by siderophore-specific periplasmic proteins, which mediate transport across the inner membrane through their cognate permeases (28, 37, 46, 47).

Almost 70% of the host iron is bound in heme (27). Thus, many bacterial pathogens secrete sphingomyelinases to lyse erythrocytes and to gain access to hemoglobin-bound heme (48–50). Hemoglobin is captured by the surface protein IsdB of *S. aureus* (51) and is then imported and degraded by other proteins of the iron-regulated surface determinant (Isd) system (52). Gram-negative bacteria often secrete proteins called hemophores, which sequester heme from host hemoproteins (42, 53). The high affinity heme uptake system Has in *Serratia* spp. utilizes the secreted hemophore HasA, which sequesters heme from host hemoproteins (54, 55). Thus, in Gram-negative bacteria hemophores, host hemoproteins or heme released from hemoglobin after proteolytic degradation are bound to specific outer membrane receptors (53). Then, heme is removed from these proteins and transported into the periplasm in a TonB-dependent manner where it is bound by heme-binding proteins and transported across the inner membrane through cognate inner membrane permeases (53).

The host proteins transferrin and lactoferrin transport iron to the cells and control the level of free iron in the blood and external secretions (56–58). These proteins constitute approximately 12% of the iron in the human body (27) and, not surprisingly, represent another iron source utilized by bacteria (30, 59). The best studied example are *Neisseria* spp. which, unlike most Gram-negative bacteria, do not produce siderophores but instead extract iron directly from serum transferrin (60). To this end *Neisseria* produce the outer membrane receptor complexes TbpA/TbpB and LbpA/LbpA (61) which bind transferrin and lactoferrin, respectively. The mechanism of TonB-dependent iron removal from transferrin by the concerted action of TbpA and TbpB has been elegantly elucidated (62). Iron is channeled through the TbpA pore, bound by periplasmic proteins and then imported across the inner membrane by ABC transporters (63).

Iron acquisition by *M. tuberculosis*

Mycobacterium tuberculosis, as most other bacterial pathogens, requires iron for growth (15, 64). For years it was believed that *M. tuberculosis* only relies on its siderophores, MBT and cMBT, for iron acquisition (64). This view was supported by observations that MBT biosynthesis is critical for growth of *M. tuberculosis* after the internal iron stores have been depleted. Carboxymycobactins are capable of removing iron from transferrin and ferritin (65) in contrast to most other bacterial siderophores (66). Consistent with this observation is the finding that a MBT synthesis mutant did not grow with human transferrin as the only iron source, demonstrating that *M. tuberculosis* has no active transferrin uptake system *in vitro* (17). *Mycobacterium tuberculosis* attracts transferrin both *in vitro* (67) and in macrophages (68, 69). However, the conclusion by Boradia *et al.* that *M. tuberculosis* can internalize human transferrin (67) is based on a flawed use of a wild-type *M. tuberculosis* strain which secretes siderophores capable of removing iron from transferrin (65). By contrast, it has been conclusively shown that siderophore-deficient *M. tuberculosis* mutants can utilize heme as an alternative iron source (17, 18).

Compared to other bacteria relatively little is known about siderophore-mediated iron acquisition by *M. tuberculosis*. Since mycobacteria have two membranes (70–72), in principle secretion and uptake mechanisms resemble more closely that of Gram-negative bacteria (73). Siderophores are synthesized by cytoplasmic synthases encoded by two *mbt* operons (74, 75). Synthesis and transport of siderophores are likely coupled (76) and depend on the membrane proteins MmpS4 and MmpS5 that are associated with the transporters MmpL4 and MmpL5 of the resistance-nodulation-cell division (RND) superfamily (76). Export of siderophores across the outer membrane probably requires an as yet unknown outer membrane channel. Secreted cMBTs bind iron, but it is unknown how they are re-captured by *M. tuberculosis* and how they cross the outer membrane. Ferric-cMBTs are transported across the inner membrane by the IrtA/IrtB protein complex (77). Iron is probably released from the imported cMBTs by a reductive mechanism rather than by enzymatic degradation (78). This mechanism leaves the siderophores intact so that they can be recycled by the export system consisting of MmpL4/MmpS4 and MmpL5/MmpS5 as recently shown (79). The type VII protein secretion system Esx-3 of *M. tuberculosis* is required for iron acquisition, but its mechanistic role is unclear (80–83). Recently, it was observed that *M. tuberculosis* releases membrane vesicles containing ferric MBTs under iron limitation (84). It has been proposed that these vesicles might be a means to share iron between *M. tuberculosis* cells; however, it is not clear what advantage these vesicles have over secreted cMBTs, which are accessible to all *M. tuberculosis* cells. Maybe the role of these vesicles is rather to gain access to host iron stored in hydrophobic environments that are not accessible for cMBT or to traverse the hydrophobic extracellular matrix of an *M. tuberculosis* biofilm as suggested previously (79)?

Even less is known about heme uptake by *M. tuberculosis*. *Mycobacterium tuberculosis* produces the heme-binding protein

Rv0203, which appears to improve but is not essential for heme utilization (18). Rv0203 was found in the culture filtrate of *M. tuberculosis* and was proposed to be a hemophore. However, Rv0203 transfers heme to the extracellular domains of the inner membrane proteins MmpL3 and MmpL11 (85, 86). This finding rather indicates a localization of Rv0203 in the periplasm. The roles of the proposed heme importers MmpL3 and MmpL11 are also unclear, since MmpL3 has been shown to export trehalose monomycolate (87, 88) and other known MmpL proteins are exporters of lipids or lipid-like molecules (76, 89–92). Intracellular heme is then degraded by the non-canonical enzyme MhuD without releasing CO. This unusual heme degradation mechanism may have evolved to avoid producing a signal for transition of *M. tuberculosis* to dormancy (93).

Regulation of iron homeostasis in *M. tuberculosis*

Iron uptake and utilization are tightly regulated by *M. tuberculosis* to avoid free iron in the cell cytoplasm. Transcriptional profiling revealed that 155 genes are differentially regulated as a result of iron availability and approximately one-third of those genes are regulated by the iron dependent regulator (IdeR) (94). In the presence of iron, IdeR binds to the so-called iron boxes at promoters and represses expression of genes for siderophore synthesis and activates genes encoding iron storage proteins, such as the bacterioferritins BfrA and BfrB (95–97). IdeR is essential for growth of *M. tuberculosis in vitro* because unregulated iron uptake increases oxidative stress and leads to accumulative self-damage eventually killing *M. tuberculosis* (98). This study also showed that IdeR is required for survival of *M. tuberculosis* in mice indicating the importance of iron homeostasis for virulence of *M. tuberculosis*.

Role of iron in tuberculosis and in virulence of *M. tuberculosis*

In the late 19th century, the French physician Armand Trousseau recognized that treating anemic tuberculosis patients with iron salts exacerbated the disease (99). These circumstantial findings have been substantiated in clinical studies (100, 101) and reproduced in model systems (102, 103). For example, an iron-rich diet increased the bacterial burden in mice infected with *M. tuberculosis* (103). Further, β-2-microglobulin-deficient mice suffer from iron overload in tissues and increased replication of *M. tuberculosis*. Treatment of these mice with lactoferrin reduced *M. tuberculosis* counts in organs establishing that iron overload represents an exacerbating factor for tuberculosis (103). Conversely, host factor polymorphisms also support the conclusion that iron availability is important in tuberculosis pathogenesis in humans. For example, mutations of the Nramp1, a divalent metal transporter expressed exclusively in phagocytic cells, have been associated with increased susceptibility to tuberculosis (104, 105) (*Fig. 1*).

These observations suggested that iron acquisition is essential for virulence of *M. tuberculosis*. However, it has been difficult to obtain conclusive experimental evidence for this hypothesis for several reasons. We have observed that *M. tuberculosis* requires siderophores to grow *in vitro* even under high iron conditions (79). This makes it impossible to obtain truly MBT-deficient mutants without constructing conditional mutants or supplementing with iron-loaded siderophores or heme (106). In addition, gene deletions in the main MBT operon (*mbtB-mbtH*) often do not disrupt the expression of downstream genes and do not fully disrupt MBT biosynthesis because the lack of individual enzymes in this pathway may be compensated for by other Mtb enzymes at a low level, in contrast to the lack of several Mbt enzymes. Such a phenomenon might be the explanation why an *mbtD::hyg* deletion completely abolished MBT production, but not the unmarked *mbtD::loxP* mutant (79). Such

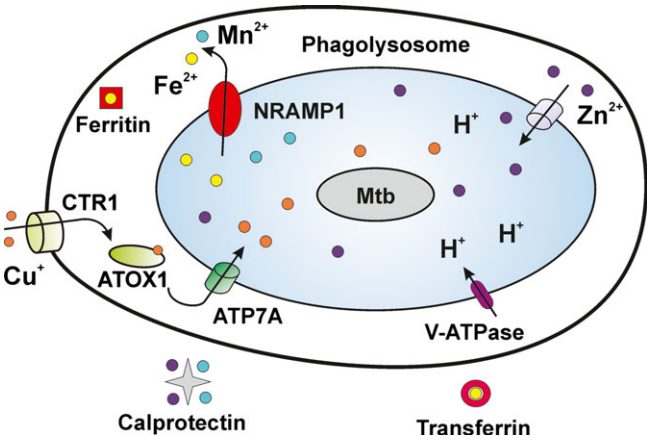

***Fig. 1.* Role of metals during infection with *Mycobacterium tuberculosis*.** CTR1 translocates Cu^{+} from the extracellular space to the cytoplasm of macrophages infected with *M. tuberculosis*. Then, Cu^{+} is bound by the chaperone ATOX1, which delivers copper to the ATP7A pump resulting in copper accumulation in the phagosome (19, 139, 174, 210). V-type ATPases and an unknown transporter pump protons and Zn^{2+}, respectively, into the phagosome (20, 180). Nramp1 (natural resistance-associated macrophage protein 1) exports Fe^{2+} and Mn^{2+} out of the phagosome. Fe^{2+} is bound by intracellular ferritin (199). The extracellular proteins transferrin sequesters iron, while calprotectin sequesters Mn^{2+} and Zn^{2+} (199). Calprotectin is secreted by neutrophils in tuberculosis granulomas (114) likely to deplete granulomas from Mn^{2+} and Zn^{2+}.

a mechanism may also explain the residual MBT production by the *M. tuberculosis mbtB* mutant (107). However, this study did show that even a reduced MBT synthesis impaired replication of *M. tuberculosis* in macrophages. Reddy *et al.* (108) also demonstrated that a mutant lacking *mbtE* did not synthesize siderophores anymore and failed to grow in low iron medium. However, this study is controversial because infection of guinea pigs with the *mbtE* mutant and wildtype *M. tuberculosis* showed similar pathology for both strains, but only wildtype *M. tuberculosis* was recovered on plates with organ homogenates (106). The inner membrane transporter IrtA/IrtB is required for efficient uptake of cMBT, but the residual cMBT uptake by the *irtAB* mutant also indicated the presence of a second transporter (77). Nevertheless, deletion of *irtAB* significantly impaired the ability of *M. tuberculosis* to grow under iron limiting conditions *in vitro* and in mice lungs, indicating that the cMBT uptake is mainly mediated by IrtAB and that the activity of IrtAB is required for full virulence of *M. tuberculosis* (77). By far the strongest *in vivo* phenotype was obtained for the *M. tuberculosis mmpS4-mmpS5* double mutant. Lack of MmpS4 and MmpS5 strongly reduced siderophore secretion and growth of *M. tuberculosis* under iron limiting conditions and made *M. tuberculosis* avirulent in mice (76). However, this virulence defect can only partially be attributed to reduced iron uptake and might, in fact, be largely caused by self-poisoning of *M. tuberculosis* by taking up active siderophores in the absence of a functional siderophore recycling system consisting of MmpS4/MmpL4 and MmpS5/MmpL5 (79). Another complication in assessing the role of iron for *M. tuberculosis in vivo* is the availability of heme as an alternate iron source in addition to the partial redundancy in siderophore uptake systems and the occurrence of secondary effects when siderophore secretion is impaired. Hence, it might be necessary to construct a conditional mutant that cannot utilize both iron sources to elucidate the real importance of iron acquisition for *M. tuberculosis in vivo*.

Role of other transition metals in virulence of *M. tuberculosis*: the case of manganese, nickel, and cobalt

Although iron is by far the best studied transition metal, other transition metals such as manganese, nickel, and cobalt are also essential micronutrients for *M. tuberculosis*. Manganese is critical for the viability and virulence of many bacterial pathogens. Emerging evidence indicates that invading microbes utilize manganese to resist the effects of host-mediated oxidative stress and this metal thus plays a significant role in adaptation of pathogenic bacteria to the human host (109). Not surprisingly, the host immune system tries to restrict the availability of both manganese and zinc in response to bacterial infections by using the chelating protein calprotectin (110–113). Interestingly, S100 proteins such as calprotectin are the dominant proteins produced by neutrophils in lung granulomas of TB patients (114), indicating that our immune system tries to sequester manganese and zinc from *M. tuberculosis* in tissues to restrict its growth and resistance to reactive oxygen intermediates (*Fig. 1*). However, direct evidence for this hypothesis is lacking. Nickel and cobalt are the two remaining out of six first-row 3*d*-block transition elements that function as inorganic co-factors in up to 25% of all proteins in cells (115). Nickel is a co-factor of the *M. tuberculosis* urease Rv1848 (116). Cobalt is required for the biosynthesis of vitamin B12 (43). The transcriptional regulators KmtR and NmtR of *M. tuberculosis* function as two nickel-cobalt sensors, further suggesting physiological significance for these ions (117). While uptake of cobalamin is utilized by *M. tuberculosis* to synthesize vitamin B12 and may contribute to *M. tuberculosis* survival in macrophages, cobalt acquisition systems are not known (118). It is apparent that our knowledge about the role of manganese, nickel, and cobalt in tuberculosis is rudimentary at best, and further studies are required in order to decipher the mechanisms involved in acquisition and utilization of these metal species.

Copper in host defense against *M. tuberculosis* and in mycobacterial virulence

The physiological roles of copper and its toxicity

Copper is a redox-active metal and, like iron, cycles mainly between two oxidative states Cu(I) (cuprous) and Cu(II) (cupric) under physiological conditions. The Cu (II)/Cu(I) redox potential in proteins is higher than that of Fe(III)/Fe(II) ranging from 250 to 750 mV enabling catalysis of oxidations using oxygen (24). This has been exploited by most living organisms, including mycobacteria and humans. One prominent example of the many known copper enzymes and proteins is the cytochrome *c* oxidase, which is a key component of aerobic respiration (119–121).

Copper also is able to engage in Fenton chemistry with hydrogen peroxide (122), an endogenous byproduct of aerobic respiration (35), to generate hydroxyl radicals in a similar manner as known for iron. Hydroxyl radicals react with most biomolecules including DNA (34) and membrane

lipids (123). DNA damage was broadly accepted as the main mechanism of copper cytotoxicity. However, a recent study (124) did not find any evidence of oxidative DNA damage in *E. coli* overloaded with copper. Since neither DNA damage nor lipid peroxidation could fully explain the bactericidal properties of copper ions, Macomber and Imlay (125) investigated the direct effect of copper overload on cellular proteins. They found that the antibacterial properties of copper ions on *E. coli* are mainly due to inhibition of intracellular dehydratases with exposed iron-sulfur clusters in a ROS-independent process (125). Copper was found to remove, as opposed to replace, Fe from iron-sulfur clusters, which further deteriorated by an undefined mechanism until only the apoenzyme remains (125, 126). In agreement with these findings, iron-sulfur cluster proteins were also targeted by copper in *B. subtilis* (127). Microarray data from *M. tuberculosis* exposed to copper also indicated damage on iron-sulfur cluster enzymes (128). Taken together copper is an essential micronutrient for most cells, but its uptake and reactivity must be strictly controlled to ensure cellular survival.

Copper homeostasis in macrophages

Eukaryotic cells including macrophages utilize an array of copper uptake, sequestration and trafficking proteins to maintain copper homeostasis and ensure that all copper ions securely reach their target sites. Divalent copper in the blood must be reduced prior to entering the cell, possibly by the action of membrane associated copper reductases (129). Cu^+ is taken up by the high affinity Cu^+ import protein CTR1 (*Fig. 1*), while copper toxicity is prevented by cytosolic metallothioneins, which sequester any surplus copper to prevent cellular damage (130, 131). Intracellular copper trafficking is mediated by chaperones which typically receive Cu^+ immediately after it enters the cell (131). The exact mechanism of copper transfer between CTR1 and cytosolic copper chaperons is unknown, but may involve glutathione (132, 133). The copper chaperon Cox17 is known to supply copper to mitochondrial cytochrome c oxidase (134) and CCS supplies cytosolic superoxide dismutase 1 (135), while ATOX1 delivers the Cu^+ ions to the copper transporter ATP7A or ATP7B of the secretory pathway for incorporation into copper requiring proteins that pass through the trans-Golgi network (e.g. lysyl oxidase, tyrosinase) (136, 137). ATP7A also translocates to the plasma membrane pumping excess cytosolic copper out of the cell (138), and to the phagosome (139).

Immunological functions of copper

In humans, nutritional or inherited copper deficiency (Menkes Syndrome) is associated with multi-system pathologies, including increased susceptibility to bacterial infections (140). Correspondingly, induced or natural copper deficiency in animals has been shown to impair the ability of macrophages and neutrophils to generate an oxidative burst and effectively kill phagocytized microbes (141, 142). Despite the long-standing observations that copper promotes a healthy immune system (143), the recognition of copper as an integral part of innate immune responses is relatively recent. Several lines of evidence now indicate that copper redistribution and mobilization in mammalian tissues and individual cells is a key immune response to bacterial infections (144–146). We previously investigated the distribution of copper in lungs of *M. tuberculosis*-infected guinea pigs and found significantly elevated copper levels in primary granulomas while the copper content in unaffected lung tissue remained low (19). Hypoxia, a hallmark of tuberculosis granulomas (147), has been shown to induce the expression of *ctr1* in human lung tissue (148) and in macrophages (149) and may constitute the signal for the copper increase at the site of *M. tuberculosis* infection. In macrophages, this phenomenon also occurs in the absence of hypoxia, where proinflammatory molecules such as INF-γ or bacterial TLR agonists (e.g. LPS) induce a similar response (139). White *et al.* (139) demonstrated that within *E. coli* infected macrophages, ATP7A translocates to the phagosomal membrane and enhances their bactericidal activity by presumably facilitating the transport of copper into the phagosome. However, *M. tuberculosis* is unique, as it has seemingly evolved to circumvent this immune response by pursuing two major strategies: impairing macrophage phagosome functions (150–153) and maintaining an extremely low intracellular copper content (19, 154).

Mycobacterial copper homeostasis and copper resistance proteins of *M. tuberculosis*

Only one copper enzyme in *M. tuberculosis* is known with relevance for both *in vitro* growth and survival in the host. The aa_3-type cytochrome *c* oxidase is a key component of aerobic respiration in the cytoplasmic membrane (155). Its two core subunits, CtaC (Rv2200c, subunit II) and CtaD (Rv3043c, subunit I), are essential for growth of *M. tuberculosis* and harbor two copper centers which are jointly responsible for the electron transfer from cytochrome *c* to dioxygen (156, 157). Surplus energy from this intramolecular

electron transfer process is used to generate a proton gradient across the cytoplasmic membrane which propels ATP synthesis (156). Interestingly, mycobacteria also have a copper-independent terminal oxidase, the cytochrome *bd* oxidase, which is critical for adaptation to an oxygen restricted environment (155, 158, 159) in which copper ions are also the most toxic to microbes (160). The switch to a copper-independent metabolism may thus also protect, at least partially, from copper-mediated toxicity when oxygen is scarce.

The stress response of *M. tuberculosis* toward copper has mainly been studied *in vitro*. Microarrays identified 30 Cu-responsive genes (128). Expression of some of these genes was also induced in macrophages and in animal models (128) suggesting that *M. tuberculosis* encounters copper toxicity in host cells. Several copper resistance mechanisms of *M. tuberculosis* have been identified. The first line of defense is the outer membrane of *M. tuberculosis* (71, 161). Copper uptake across mycobacterial outer membranes is controlled by channel proteins as shown for *M. tuberculosis* and MspA in *M. smegmatis* (162). The inner membrane of *M. tuberculosis* hosts CtpV, a P-ATPase cation transporter that likely acts as a copper efflux pump (154, 163). However, CtpV deletion does not result in virulence defects in mice perhaps because *M. tuberculosis* has the capacity to at least partially compensate for the loss of CtpV by expression of alternative metal efflux pumps (154, 163). Indeed, 3 out of the 12 P-type ATPases (CtpA, CtpB, CtpV) of *M. tuberculosis* have predicted preference for Cu, as discussed below (163). Another membrane protein, MctB, also decreases intracellular copper levels and is required for full *M. tuberculosis* copper resistance and virulence in mice and guinea pigs (19, 164). However, its exact function in copper homeostasis remains undefined and its precise location within the cell envelope is not known (22). In addition to controlling copper uptake by membrane proteins, *M. tuberculosis* attempts to detoxify and sequester copper ions. The periplasmic multicopper oxidase MmcO is a homolog of *E. coli* CueO and probably oxidizes Cu(I) to the less toxic Cu(II) (23). *MmcO* expression is induced by copper, though a virulent clinical strain of *M. tuberculosis* lacks the gene (165), suggesting a redundancy with other copper resistance mechanisms. The metallothionein MymT binds multiple copper ions within the cytoplasm (21). Finally, *M. tuberculosis* may employ yet unknown resistance mechanisms which are regulated on a transcriptional level by CsoR and RicR. CsoR has a very high affinity for copper ions ($K \geq 10^{19}\ M^{-1}$) which allows *M. tuberculosis* to respond to small amounts of free Cu(I) and to induce transcription of the copper-sensitive operon *cso* (166). The *cso* operon encodes CsoR itself, CtpV, and two proteins of unknown function (Rv0978, Rv0980). Recently, RicR was identified as an additional regulator in *M. tuberculosis* that also dissociates from its cognate DNA-binding sites upon binding copper (167). The *ric* regulon comprises *ricR*, *mymT*, two genes encoding the predicted membrane proteins LpqS and Rv2963, and the *socAB* locus of unknown function (167). Interestingly, absence of any single copper resistance gene controlled by RicR is not sufficient to induce copper susceptibility, but mutation of the copper-binding residue in RicR, and thus ablation of its copper-sensing ability, increases copper susceptibility and reduces virulence of *M. tuberculosis* (168).

Role of copper in the phagosome

The majority of these mechanisms were studied in bacterial cell culture and may not accurately reflect the situation in macrophages. Bioavailability of copper and its reactivity are dependent on many factors including medium composition and preparation, pH, and redox status (160, 169, 170). For example, *E. coli* is more susceptible to copper under anaerobic conditions resembling a reducing environment while aerobically cultured bacteria are quite resilient (160). Similarly, *E. coli* appears to be more sensitive to copper in minimal medium (MIC <0.01 mM) than when grown in rich medium (MIC >1 mM) (171, 172), which, also holds true for *M. tuberculosis* (154, 168, 173). One prerequisite for copper toxicity to take place in the phagosome is therefore the presence of a chemical environment that promotes copper toxicity at concentrations reported for phagosomes (0.02–0.4 mM) (174). In addition, *E. coli* transcriptionally and metabolically adapts to copper *in vitro*, by using alternative enzymes or activating pathways that are less affected by copper. However, such adaptation may not be possible *in vivo* due to nutrient starvation, energy limitation and the abundance of antibacterial molecules in the phagosome (e.g. metal ions, ROS, acidity, antibacterial peptides).

In the phagosome, it is likely that copper ions encounter hydrogen peroxide outside of the bacterial cell. NADPH oxidase, a membrane integral protein that is recruited to the phagosomal membrane, generates superoxide radical anion from molecular oxygen (175, 176). The dismutation of the superoxide anion generates hydrogen peroxide in the lumen of the phagosome which could potentially provide the means for copper ions to undergo Fenton chemistry as described above (146). The inflicted oxidative damage on lipids may not kill the bacteria per se but could prime the bacterial cell for subsequent destruction by other phagosomal

functions. The potential synergism of bactericidal mechanisms in the phagosome, e.g. between copper overload and oxidative burst (146), may also prevent to experimentally determine the relevance of individual resistance mechanisms *in vivo*.

Zinc in host defense against *M. tuberculosis* and in mycobacterial virulence

Zinc toxicity results from replacing other cations in essential enzymes, thereby blocking their activity (177). In addition, Zn^{2+} competes with Mn^{2+} uptake systems, leading to Mn^{2+} deficiency. For instance, the *Streptococcus pneumoniae* Mn^{2+} importer PsaA is blocked by Zn^{2+}, inducing Mn^{2+} deprivation and increased sensitivity to oxidative stress (178, 179). Whether Zn^{2+} can inhibit the putative Mn^{2+} importer MntH (Rv0924c) of *M. tuberculosis* remains to be evaluated.

In addition to copper, a novel host defense mechanism against infections relying on intoxicating microbes inside phagosomes through zinc overload has recently been reported. We (20, 180) have shown that zinc accumulates in the mycobacterial phagosome as well as in vacuoles containing other microbes, such as *E. coli*, during infection, and that the P-ATPase CtpC is required for optimal intracellular growth of *M. tuberculosis*. Interestingly, we showed that zinc also accumulates in phago-lysosomes containing non-pathogenic species such as *E. coli* and that a mutant of *E. coli* in the well-characterized zinc efflux P-ATPase ZntA was killed faster than its wild-type counterpart in macrophages.

The total amount of zinc in living organisms is highly regulated (0.1–0.5 mM representing the so-called 'zinc quota') and because of its toxicity, free zinc is present in very limited amounts in cells, most zinc atoms being bound to proteins such as metallothioneins, ribosomes etc., referred to as the 'zinc proteome'. In the presence of an excess of free zinc, eukaryotic cells react by translocating the zinc-sensing metal transcription factor MTF-1 to the nucleus, which induces expression of zinc detoxification genes, such as the metallothionein-encoding genes *mt1* and *mt2*, and the zinc efflux transporter-encoding gene *znt1*/*slc30a1* (181).

The observation of such a signature of zinc stress in *M. tuberculosis*-infected macrophages prompted us to evaluate whether free zinc was present in excess amounts in infected cells, which was confirmed by confocal microscopy (20). However, zinc labeling was clearly concentrated to small intracellular compartments in infected macrophages. Such compartments are referred to as 'zincosomes' in the literature (182). They may allow zinc storage and buffering, thereby avoiding zinc to be present in excess in the cytosol (183). Zincosomes have been suggested to represent a subset of the late endosomal pool. Indeed, most zincosomes stain positive for the late endosomal and lysosomal markers LAMP-1 and Cathepsin D (20). However, we also observed a fraction of zincosomes staining positive for the early endosomal marker Rab5, strongly suggesting that zincosomes span over the entire endocytic pathway. Our results suggest that free zinc is released from an intracellular pool rather than being influxed from the outside of the cells in *M. tuberculosis*-infected macrophages. Release of zinc from intracellular zinc-containing proteins is blocked by chemical inhibitors of the NADPH oxidase (e.g. apocynin) implying a role of oxygen radicals generated upon infection in this immune response (20). However, the exact origin of the free zinc fraction observed in infected macrophages, the signals leading to zinc release, the transporters involved in zinc relocalization to the zincosomes, and most importantly the mechanisms and putative transporters implicated in zinc accumulation in phagosomes are unknown. ZnT1-10 (SLC30A1-10) form a family of eukaryotic zinc transporters that are expressed in various cells and tissues, and that localize to the plasma membrane and intracellular vesicles, thereby allowing zinc efflux from the cytosol to the extracellular milieu, or zinc influx from the cytosol to the lumen of intracellular compartments (181). It is anticipated that zinc mobilization to zincosomes and phagosomes in macrophages is due, at least in part, to one or more ZnT transporter(s), which remains to be further explored.

Equally important will be to understand the exact function of CtpC, and possibly other *M. tuberculosis* P-ATPases, in mycobacterial resistance to zinc intoxication. In this regard, the putative CtpC cognate metallochaperone Rv3269 is highly intriguing and its function should be further dissected. Rv3269 is a small putative peptide of 93 amino acid residues, with a Val^{5}-Tyr^{24} putative transmembrane (TM) domain, and a cytoplasmic Asp^{87}-Leu-His-Asp-His-Asp-His^{93} C-terminal domain. The facts that *rv3269* is induced together with *ctpC* in response to zinc (20) and that the two genes are encoded in an operon strongly suggest a common function. It is tempting to speculate that Rv3269 binds Zn^{2+} through its C-terminal domain and transfer the metal ion to CtpC for active efflux. Finally, the CtpG-encoding gene is also induced by Zn^{2+} stress (20) indicating that this transporter also contributes to zinc efflux.

Metal efflux in *M. tuberculosis*

In prokaryotes, resistance to metal toxicity heavily relies on efflux systems and this appears to be the case for *M. tuberculosis* as well. Metal efflux systems belong to three main families: heavy metal efflux members of the RND superfamily (HME-RND), the cation diffusion facilitators (CDF) family, and the P-type ATPase family (184). Gram-negative bacteria frequently expel toxic metal ions through tripartite efflux pumps of the RND superfamily that form a complex with a periplasmic membrane fusion protein and an outer membrane channel spanning both the inner and outer membranes. For example, the CusCBA efflux system extrudes biocidal Cu(I) ions (185, 186). This efflux system is capable of picking up the metal ions from both the periplasm and the cytoplasm and uses methionine residues to export Cu(I) ions (187). By contrast, we do not know any outer membrane component of metal efflux systems in *M. tuberculosis* and we are only beginning to identify inner membrane efflux pumps and to determine their metal specificity. The *M. tuberculosis* genome (188) contains no member of the HME-RND family and only one putative CDF transporter (Rv2025c). Expression of *rv2025c* is repressed by the transcriptional repressor KmtR (Rv0827c) and is induced by Ni^{2+} and Co^{2+}, suggesting that Rv2025c transports Ni^{2+} and Co^{2+} (117). In addition, *M. tuberculosis* contains no member of the recently discovered MntX family involved in Mn^{2+} efflux (189) and no close homolog of ZntB, a member of the CorA family shown to mediate Zn^{2+} efflux in *Salmonella* (190). However, the *M. tuberculosis* genome codes for the striking number of 12 P-type ATPases (named Ctp for cation-transporting protein), whose substrate specificities are still partially unknown (180, 191). Ions are transported by P-ATPases by coupling ATP hydrolysis at the cytoplasmic domain with ion translocation across the inner membrane through the TM domain of the transporter. This mechanism is well conserved throughout evolution. The *M. tuberculosis* P-ATPases are members of different families. While KdpB (Rv1030) is a putative P_{1A}-type ATPase K^+ transporter, CtpA (Rv0092), CtpB (Rv0103c), CtpC (Rv3270), CtpD (Rv1469), CtpG (Rv1992c), CtpJ (Rv3743c), and CtpV (Rv0969) are P_{1B}-ATPases involved in the transport of metal cations. CtpF (Rv1997c) is a putative P_{2A}-type Ca^{2+} transporter. CtpE (Rv0908), CtpH (Rv0425c) and CtpI (Rv0107c) constitute atypical P-ATPases with no substrate prediction. CtpE, CtpF, CtpH, and CtpI exhibit a Pro-Glu-Gly-Leu-(Pro/Val) motif in the membrane-spanning helix located upstream the phosphorylation site. This motif is found in all Ca^{2+}-ATPases where it is part of the calcium transport site. Interestingly, upstream of the *ctpC*, *ctpG*, and *ctpV* genes are genes encoding putative metallochaperones (Rv3269, Rv1993c, and Rv0968) that might play a part in metal selectivity and transport mechanism of their cognate P-type ATPase, as recently demonstrated for a similar transport system in *S. pneumoniae* (192).

Inference on selectivity of P-type ATPases for metal ions is difficult, and relies on similarities to known transporters, on the presence of conserved metal-binding motifs, the function of neighboring genes and on gene regulation by metal ions. Metal transporting P_{1B}-ATPases have been classified into five subfamilies on the basis of sequence homology (193). Interestingly, this study revealed that each subfamily possesses conserved amino acids in TM helices 6, 7, and 8, likely to be involved in metal coordination. According to these criteria CtpA, CtpB, and CtpV may be part of the P_{1B1}-type subfamily of Cu^+-ATPases, while CtpD and CtpJ belong to the P_{1B4}-type subfamily of Co^{2+}-ATPases (194, 195). This classification of CtpJ is in agreement with the regulation of this transporter by the Ni^{2+}/Co^{2+}-sensing DNA-binding repressor NmtR (196). CtpG is embedded in an operon together with the Cd^{2+}/Pb^{2+}-sensing regulator CmtR (Rv1994c), suggesting CtpG is a Cd^{2+}/Pb^{2+} efflux transporter. The hypothesis that CtpV might efflux copper is supported by the facts that (i) the *ctpV* gene, together with that of its cognate regulator- and putative metallochaperone-encoding genes *csoR* and Rv0968, is induced in response to Cu^+ excess (128); and (ii) a *ctpV*-null mutant of *M. tuberculosis* is highly sensitive to Cu^+ (197). Similarly an excess of Co^{2+} induces the CtpD- and CtpJ-encoding genes, and mycobacterial mutants inactivated in these transporters accumulate Co^{2+} (195), suggesting CtpD and CtpJ transport Co^{2+}.

While we and others found the *ctpC* gene is strongly induced by Zn^{2+}, and a *ctpC*-null mutant of *M. tuberculosis* is highly sensitive to Zn^{2+} intoxication (20, 198), kinetics using recombinant CtpC suggested the protein might transport preferentially Mn^{2+} over Zn^{2+} (198). This apparent discrepancy might be explained by the fact that the study by Padilla-Benavides *et al.* (198) did not include the putative CtpC metallochaperone Rv3269 in their *in vitro* systems. Rv3269 contains a clear putative zinc-binding motif (DLHDHDH) in its C-terminus end, which might confer zinc-specificity to CtpC.

Recent studies suggested a role for CtpC, CtpD, and CtpV, as well as for other metal efflux or resistance systems in *M. tuberculosis* virulence, suggesting that in addition to metal

withholding, mammalian phagocytes exploit the toxic properties of transition metals to control bacterial infections (199).

Novel intervention strategies to enhance metal toxicity against *M. tuberculosis*: the case of copper

Early experiments showed that the anti-mycobacterial activity of isoniazid, a main drug in current tuberculosis chemotherapeutic regimens, was enhanced by copper binding indicating a synergistic effect (200, 201). These findings suggested that it might be possible to identify novel copper-chelating compounds with anti-mycobacterial activities. Indeed, a copper-dependent drug screen identified copper complexes of bis-thiosemicarbazones with activities against *M. tuberculosis* (173). The minimal inhibitory concentration of GTSM [glyoxalbis(N(4)-methyl-3-thiosemicarbazone] was ~300 nM and killed non-replicating *M. tuberculosis* at a concentration of 2.5 μM. An interesting feature of bis-thiosemicarbazones copper complexes is their ability to accumulate in hypoxic tissues (202, 203). While this ability is currently exploited for diagnosis and potential treatment of certain cancers and neurodegenerative diseases (204, 205), it could also be beneficial in tuberculosis chemotherapy as hypoxia is a well-known condition of infected lung tissue (147). The GTSM-copper complex also inhibits the growth of methicillin-resistant *S. aureus* and *Neisseria gonorrhoeae* in a copper-dependent manner (206, 207). By contrast, zinc and iron, which are the two most abundant transition metals in the human body (208), did not impede Cu(II)-GTSM activity (206), suggesting specificity of copper binding by GTSM *in vivo*. McEwan and coworkers (207) found that copper overload did not occur in GTSM-treated *N. gonorrhoeae* cells which is consistent with the fact that the active concentration of 30 nM GTSM is too low to significantly change the average cellular copper content of 10 μM. Instead, the authors showed that Cu(II)GTSM specifically targets NADH and succinate dehydrogenase, two respiratory enzymes which are no longer able to maintain electron flow to terminal oxidases upon binding of the copper complex and its reduction by the enzyme (207). For the first time, this study revealed that copper complexes with small molecules act on defined bacterial targets and by novel mechanisms distinct from the general toxicity of free copper ions. These studies indicate that it is possible to enhance the toxicity of metals to kill bacterial pathogens by selectively targeting essential cellular processes. Whether similar strategies can be used to enhance the toxicity of other metal ions such as zinc remains to be explored.

Conclusions and perspectives

A key trait of *M. tuberculosis* is to exploit and manipulate metal cation trafficking inside infected macrophages to ensure survival and replication inside the phagosome. However, we are just at the beginning to discover all the components of metal acquisition and detoxification systems of *M. tuberculosis*. We do not know how these proteins interact with each other to guide metal cations in both uptake and efflux processes. A better understanding of metal transport processes in *M. tuberculosis* is not only important for deciphering the physiology of *M. tuberculosis in vivo*, but will also likely provide a plethora of novel molecular mechanisms as apparent from the few known metal-related transport systems (18, 76, 79). Occasionally, knowledge of these pathways may reveal an unexpected vulnerability of the tuberculosis bacillus. A recent example is that blocking siderophore export leads to self-poisoning of *M. tuberculosis* (79) and renders it completely avirulent (76). Conversely, the mechanisms by which metal is transported into or out of the phagosome in macrophages and the signals controlling these events are poorly understood. However, these mechanisms might offer avenues for novel anti-infective approaches, which are urgently needed considering the failing existing tuberculosis chemotherapeutic regimens as recently pointed out by Nathan (209). First, metal-targeted nutritional immunity (199) against *M. tuberculosis* could be enhanced by promoting metal depletion through stimulating transporter translocation to the phagosome and utilizing chelators combined with dietary changes. Second, the metal defense systems of *M. tuberculosis* could be targeted by novel drugs to enhance the susceptibility of *M. tuberculosis* against copper and zinc. Third, it is possible to enhance the toxicity of metals utilized by the immune system to kill bacterial pathogens as shown in a novel drug-screening approach which identified copper-boosting compounds effective against replicating and non-replicating *M. tuberculosis* strains (173). Hopefully, the fascinating recent discoveries of new metal homeostasis mechanisms both in *M. tuberculosis* and in macrophages as described in this review will stimulate more efforts to understand the battle for metals between *M. tuberculosis* and the host.

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