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REVIEW

Nutritional Immunity and Fungal Pathogens: A New Role for Manganese

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Abstract

Purpose of Review Copper, zinc, iron, and manganese are essential micronutrients for all living organisms. Microbial pathogens must acquire these elements from their host. Through a process termed nutritional immunity, animal hosts seek to withhold these vital nutrients from the microbe and the competition for metals can influence survival outcomes during infection. Much is known about the battle for iron, copper, and zinc during fungal infections, but a picture is just now beginning to emerge for manganese.

Recent Findings Pathogenic fungi utilize manganese for antioxidant defense, cell wall construction, morphogenesis, and survival in animal and plant hosts. The animal host can limit manganese availability for invading fungi at the macrophage, neutrophil, and whole tissue levels.

Summary Here, we review the role of manganese as an essential nutrient for pathogenic fungi and the ways an animal host can withhold this vital metal from infectious fungi of clinical and agricultural importance.

Keywords Fungal pathogenesis · Metals · Manganese · Mannosylation · Infection

Introduction

Metals such as Fe, Cu, Zn, and Mn play important roles in biology and serve as co-factors for nearly half of all enzymes [1]. Both participants in infection, the host and the pathogen, must acquire these micronutrients to perform necessary cellular functions. While animal hosts obtain these nutrients through diet, pathogens must scavenge trace metal nutrients from their host. The mammalian host exploits this nutritional dependence of the microbial pathogen and limits metal availability at sites of infection to effectively starve the invaders and impede their growth. Such regulation of metal availability by the host is known as nutritional immunity [2•]. Successful pathogens have evolved with specialized mechanisms to tolerate changes in metal availability. In the case of fungal pathogens, there have been many reports of the battle for micronutrients Fe, Zn, and Cu at the host–pathogen interface, and several excellent reviews have been written on the topic [3–5]. Comparatively, little, however, is known about Mn, and until very recently, an essential role for Mn in fungal pathogenesis had not been characterized. Here, we provide an overview of Mn as an essential micronutrient for fungal growth and fitness in the mammalian host and describe ways the host itself can manipulate this metal at the fungal infection battleground.

Manganese as a Micronutrient for Fungi and the Hosts They Infect

Manganese is the 12th most abundant element in the earth's crust and accumulates in biological systems as divalent and trivalent Mn^{+2} and Mn^{+3}. Mn binds selectively to oxygen and imidazolate nitrogen ligands and therefore coordinates well in enzyme active sites harboring aspartates, glutamates, and histidines. Because of its participation in redox chemistry, Mn serves as an excellent co-factor for enzymes involved in oxygen chemistry. Mn can also serve in non-redox catalytic roles for a variety of enzymes involved in metabolism and signaling. In fungi and animals, the best-characterized

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roles of Mn as a micronutrient involve the protein families of superoxide dismutases and protein glycosyltransferases.

Superoxide dismutases (SODs) are antioxidant metalloenzymes that disproportionate superoxide, a potentially toxic-free radical and reactive oxygen species (ROS) generated by metabolism. Fungi and animals typically use the Mn- and Cu-containing enzymes. Of these, a Mn-SOD generally resides in the mitochondrial matrix (so-called SOD2) to remove electron transport chain–generated superoxide, while the cytosol contains a Cu- and Zn-containing SOD1 [6]. Certain pathogenic fungi such as the opportunistic fungal pathogen *Candida albicans* uniquely express a second Mn requiring SOD3 in the cytosol that represents a clever adaptation to nutritional immunity for Cu. When *C. albicans* is limited for Cu as occurs in the kidney during infection, the fungus substitutes its Cu/Zn SOD1 with Mn-SOD3 to maintain antioxidant protection [7, 8].

Protein glycosyltransferases that reside in the Golgi decorate proteins with chains of glucose, galactose, or mannose, and this broad class of enzymes is Mn-dependent [9]. Mannosylation is prevalent among fungi, and mannosylated proteins at the cell surface create a thick mannose layer that represents the outer coat of the fungal cell wall. The Mn-dependent mannosyltransferases (MNTs) that create these elaborate chains of mannose are essential for the virulence of fungal pathogens [10, 11]. In addition to the primary roles of Mn in fungal protein mannosylation and antioxidant function, a number of kinases and phosphatases use Mn (or Mg) as co-factors [9, 12], and Mn is also believed to be the physiological substrate of the TOR kinase used in nutrient signaling [13••].

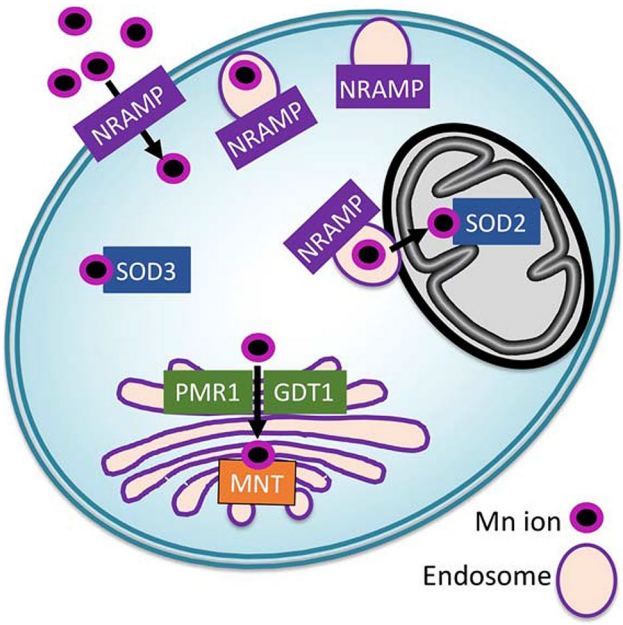

Fig. 1 Mn transport and trafficking in a fungal cell: Mn ion uptake is mediated by NRAMP Mn transporters that based on studies in *S. cerevisiae*, localize to the cell surface and endosomes. The Mn is taken up by the Golgi through Mn and Ca transporters PMR1 and GDT1 that activate Mn requiring mannosyl transferases (MNT) in the secretory pathway. Mn is also delivered to the mitochondria to activate Mn-containing SOD2. The mechanism may involve an interaction with NRAMP-containing endosomes (see main text). In certain fungi (e.g., *C. albicans*), Mn is delivered to the cytosol to activate a cytosolic Mn-SOD3

Mn Uptake and Homeostasis: Lessons Learned from Bakers' Yeast

By far, the vast majority of what is known about Mn in fungi stems from older studies in bakers' yeast *Saccharomyces cerevisiae.* Mn uptake in *S. cerevisiae* is largely accomplished by NRAMP (natural resistance-associated macrophage protein, see ahead) divalent metal transporters. Across eukaryotes and bacteria, NRAMP transporters co-transport divalent metals such as Co^{2+}, Fe^{2+}, Cd^{2+}, Mn^{2+}, and Zn^{2+} together with protons [14•]. In *S. cerevisiae*, the SMF1 and SMF2 NRAMP transporters specifically function in Mn uptake and utilization. (Note: The name SMF derives from its original isolation as a suppressor of *mif1* mutant affecting mitochondrial protein processing [15].) *S. cerevisiae* SMF1 and SMF2 have been localized to the cell surface and endosomes where they function in high affinity cell surface uptake of Mn that is then delivered to Mn-SOD2 in the mitochondria and Mn-MNTs in the Golgi (Fig. 1) [16–19]. The only other source of Mn for *S. cerevisiae* is the Mn-phosphate transporter PHO84 that operates under low-affinity (high Mn) conditions [20, 21].

S. cerevisiae SMF1 and SMF2 are negatively regulated by Mn ions in order to prevent Mn hyperaccumulation when the metal is abundant and to maximize Mn uptake when the metal is low. Such negative feedback control of metal transport is not unique to Mn and the SMF transporters. Fungal transporters for Zn, Cu, and Fe are all regulated by their cognate metal substrate at the transcriptional level using Zn, Cu, or Fe sensing transcription factors [3–5]. However, there is no known trans-regulator for Mn in yeast species, and SMF1 and SMF2 transporters are not regulated at the mRNA level. Instead, Mn regulates SMF1 and SMF2 at the post-translational level through changes in SMF protein localization and protein turnover. Under Mn-replete conditions, the transporters are targeted directly to the vacuole for degradation. Under Mn starvation conditions, the two proteins are diverted from the vacuole and accumulate at the cell surface and endosomes to facilitate Mn uptake and utilization. The sensing of Mn and control of SMF1 and SMF2 localization are accomplished by BSD2, an adaptor for the RSP5 ubiquitin ligase. BSD2 in the endoplasmic reticulum detects conformations in SMF1 that alter with Mn,

and through ubiquitination of the metal transporter, SMF1 is directed to the vacuole for degradation [16, 17, 19, 22–26].

In addition to Mn uptake at the cell surface, Mn needs to be transported into the Golgi for activation of MNTs, and this is accomplished in part by a Mn^{2+} and Ca^{2+} transporting ATPase first identified in *S. cerevisiae* as PMR1 (for plasma membrane–related ATPase) [27, 28]. Loss of PMR1 results in defective protein mannosylation without impaired mitochondrial Mn SOD2, in addition to hyperaccumulation of Mn in the cytosol. These phenotypes are consistent with a role for PMR1 in delivering Mn specifically from the cytosol to the Golgi (Fig. 1) [18, 27–29]. More recently, a second Golgi transporter has been identified that functions in Mn delivery to the Golgi for protein mannosylation, namely yeast GDT1, a proton and Ca/Mn exchanger [30, 31•]. Yeast GDT1 appears to serve as a backup to PMR1 for supplying the secretory pathway with Mn, as well as Ca (Fig. 1).

Regarding mitochondrial uptake of Mn, we previously identified mitochondrial solute transporter MTM1 as necessary for Mn incorporation into SOD2 [32]. However, MTM1 was subsequently found to function in mitochondrial homeostasis of Fe, not Mn, and yeast mutants for *mtm1* mis-incorporate Fe into the active site of SOD2 rather than Mn [33, 34]. How then does Mn reach the mitochondria for activation of Mn SOD2? Interestingly, studies in mammalian cells have implicated a NRAMP transporter in the process, namely DMT1, that like *S. cerevisiae* SMF1 and SMF2 localizes to the cell surface and endosomes. DMT1-containing endosomes can physically interact with mitochondria and deliver Mn [35, 36, 37•]. A similar scenario may occur in fungi with SMF1- or SMF2-containing endosomes (depicted in Fig. 1).

Conserved Mn Trafficking Pathways Across the Fungal Kingdom

Many of the same aforementioned Mn transport and trafficking pathways first discovered in *S. cerevisiae* have recently been identified in diverse fungal species, from environmental non-pathogenic species to pathogens that infect plants and animals. Below, we highlight commonalities identified in NRAMP transporters for Mn and in the Golgi Mn transport systems involving PMR1 and GDT1.

By genome inspections, the vast majority of fungi examined have one to five NRAMP candidates, exceptions include plant pathogens *Magnaporthe grisea* and *Alternaria brassicicola* [38]. The human pulmonary pathogen *Cryptococcus neoformans* contains a single NRAMP, denoted Cramp, that when expressed in oocytes, exhibits Mn uptake activity and is therefore likely to be a Mn transporter for the fungus. However, Cramp also has the capacity to transport Fe, Co, and Ni in this oocyte system [39], and the precise metal substrate in *C. neoformans* cells warrants investigation. Indeed, NRAMP transporters from bacteria to humans can display a great deal of promiscuity in their metal ion substrate, and it is often difficult to predict the physiological metal for transport [37•]. Perhaps, the best evidence for Mn selectivity in fungal NRAMPs has emerged through studies of the single NRAMP in filamentous *Aspergillus* sp and a pair of NRAMPs in *C. albicans.* In pathogenic *Aspergillus niger*, loss of the single NRAMP DmtA drastically reduced Mn uptake and resulted in growth defects that were rescued by supplements of Mn salts [40]. Deletion of the analogous *AoNramp1* in the non-pathogenic *Aspergillus oryzae* also resulted in growth deficiencies correctable by Mn or Zn supplements [41]. *C. albicans* contains four NRAMPs: SMF3 for vacuolar Fe transport [42], SMF11 of unknown function, and SMF12 and SMF13 that operate in Mn transport [43••, 44•]. Loss of either SMF12 or SMF13 effects a drastic reduction in total cellular Mn accumulation [43••, 44•]. The activity of both the mitochondrial MnSOD2 and the cytosolic MnSOD3 are inhibited, as are MNTs in the Golgi [43••]. Total cell surface phosphomannans are decreased, and protein mannosylation is severely impaired, including that of a vacuolar acid phosphatase as well as a cell wall Cu-containing superoxide dismutase (SOD5) needed for antioxidant defense [43••]. Interestingly, mannosylation is not totally eliminated, but the size of the mannose chains on cell wall proteins such as SOD5 is greatly reduced. *C. albicans* SMF12 and SMF13 appear to be specific for Mn, as all the defects associated with loss of these transporters were rescuable by supplements of Mn, but not other metals [43••]. Furthermore, mutations in *SMF12* and *SMF13* resulted in no loss of Fe, Zn, or Cu accumulation in *C. albicans*, only Mn was impacted [43••].

As mentioned above, the Mn NRAMP transporters in *S. cerevisiae* are negatively regulated by Mn through degradation in the vacuole involving BSD2 and ubiquitination [16, 17, 19, 22–26]. It is not currently clear whether NRAMPs in other fungal species are similarly regulated at the post-translational level. In *Aspergillus nidulans*, the BsdA homologue has been shown to target misfolded proteins to the vacuole for degradation, and the metal-bound forms of NRAMP transporters may be one such cargo [45].

Are the NRAMPs the only source of fungal Mn? With both *C. albicans* and *A. niger*, the defects associated with loss of the Mn transporting NRAMPs can be rescued by supplementation with high levels of Mn [40, 43••], implying a second Mn uptake system. It was proposed that the *A. niger* homologue to *S. cerevisiae* PHO84 (metal-phosphate

transporter) accounts for this low-affinity Mn uptake, although this has yet to be verified [40]. In *C. albicans*, mutants of *pho84* showed no deficits in Mn uptake in strains that express SMF12 and SMF13 [46], yet it remains possible that PHO84 becomes operative for Mn uptake when SMF12 and SMF13 are defective.

The PMR1 (Mn and Ca P-type ATPase) and GDT1 (Mn and Ca/proton exchanger) transport systems for delivering Mn to the Golgi for MNTs are also conserved across fungi. In *C. albicans*, loss of PMR1 causes decreases in cell surface phosphomannans and in mannosylation of acid phosphatase [47], similar to the Mn deficiency phenotypes of *C. albicans smf12* and *smf13* mutants [43••]. In the plant pathogen *Aspergillus flavus*, mutants of *PMR1* and *GDT1* exhibit defects in protein mannosylation [48••], and an analogous role for PMR1 and GDT1 in Golgi Mn uptake was described for the plant pathogen *Fusarium graminearum* [49•]. While PMR1 and GDT1 are predicted to transport Ca as well as Mn into the Golgi, the protein mannosylation defects associated with loss of these transporters were rescued by Mn supplements in *A. flavus* [48••], strongly pointing to a role for Mn transport in protein mannosylation.

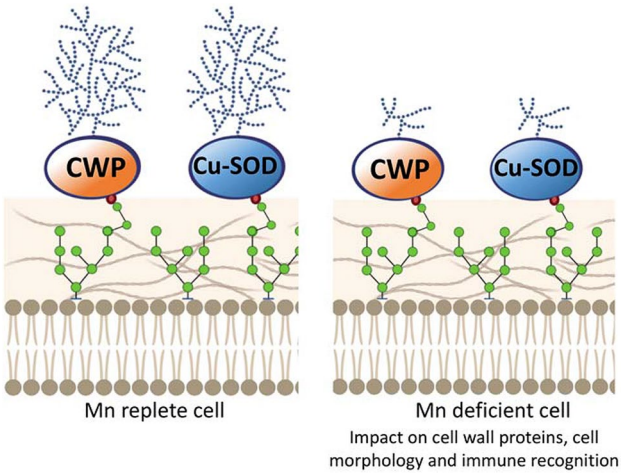

Fig. 2 The effects of fungal Mn deficiency on the cell wall. Shown are the layers of the fungal cell wall atop the plasma membrane lipid bilayer. The fungal cell wall can be comprised of inner beta-glucan (green balls) and chitin (brown horizontal lines) layers and an outer mannose layer (blue) derived from heavily mannosylated cell wall proteins (CWP) such as Cu-only superoxide dismutases (Cu-SOD). Left—under Mn-replete conditions the fungal cell wall is intact. Right—under Mn starvation conditions, the loss in activity of mannosyl transferases results in very short mannose chains on cell wall proteins and an attenuated mannose layer. The potential implications of such Mn deficiency are listed

The Role of Fungal Mn in Cell Wall Integrity, Morphogenesis, and Pathogenesis

The fungal cell wall maintains cell shape and integrity and helps accommodate morphological changes that occur during fungal differentiation and filamentation. With pathogenic fungi, the cell wall layers are important for immune recognition [50••, 51, 52]. The wall is typically composed of an inner layer of beta-glucan and chitin covered by an outer mannose layer derived from mannosylated cell wall proteins (depicted in Fig. 2). With the aforementioned role of Mn in protein mannosylation, any disruptions in Mn homeostasis could potentially impact cell wall integrity, morphogenesis, and pathogenesis. Below, we provide examples to support this role for Mn in fungi.

Several studies have linked Mn to fungal cell wall maintenance. During Mn deficiency, the outer mannose layer can become thin as indicated by the dramatic loss in cell surface phosphomannans and short-chain mannosylation of cell wall proteins seen with *C. albicans smf12* and *smf13* mutants (Fig. 2) [43••]. Additionally, *pmr1* mutants of *C. albicans*, *Aspergillus fumigatus*, and *A. flavus* show changes in cell wall thickness, an increased hypersensitivity to cell wall perturbing agents, and elevated beta-glucan and chitin content, perhaps to compensate for losses in the mannose layer [47, 48••, 53].

Regarding the role of Mn in differentiation and morphogenesis, mutants of Mn NRAMP transporters in *A. niger* and *A. oryzae* show defects in germination and hyphal morphology [40, 41], and *C. albicans smf12* and *smf13* mutants form abnormal hyphae, a defect that is corrected by Mn supplementation to the growth media [43••]. PMR1 of the plant pathogen *Magnaporthe oryzae* is essential for morphogenesis [54], and mutants of *pmr1* and *gdt1* in *F. graminearum* and *A. flavus* likewise show defects in morphology that are rescued by high Mn supplementation [48••, 49•]. Even Mn in the growth media can impact the morphology of wild-type fungi, as has been reported with filamentation of *Rhizophagus irregularis* [55] and biofilm formation in *Candida parapsilosis* [56].

With several pathogenic species, morphogenesis and differentiation are keys for fungal invasion of cells and tissues. Accordingly, mutants of Mn homeostasis have displayed losses in virulence with both plant and animal pathogens. *C. albicans* mutants of *smf12*, *smf13*, and *pmr1* show decreased virulence both in a mouse model for disseminated candidiasis [43••, 47] and in an insect model for fungal infection [44•]. In addition, *pmr1* mutants in the plant pathogens *Fusarium graminearum* and *A. flavus* have exhibited decreased virulence in plant infection models, and with *A. flavus*, this virulence defect was rescued by high Mn [48••, 49•]. Altogether these studies underscore the notion that proper Mn homeostasis is essential for fungal survival during infection.

Manganese on the Host Side of the Infection Battleground—Nutritional Immunity for Manganese

With the importance of Mn in fungal growth and pathogenesis, host manipulation of this metal may greatly influence outcomes in fungal infections. Here, we review what is currently known about host nutritional immunity for Mn in animals as applies to pathogenic fungi.

Macrophages can withhold Mn from invading microbes once they are engulfed into the phagolysosome. Pathogens housed in phagolysosomes are then attacked by a myriad of harsh conditions including hydrolytic enzymes, oxidative stress conditions, and acidic pH. Pathogens face Mn and Fe starvation in this compartment through the action of NRAMP1 [57]. NRAMP1 in fact is the prototype of the NRAMP family of divalent metal transporters named for "natural resistance-associated macrophage protein" or for its ability to enhance mouse resistance to microbial infection [57]. NRAMP1 is effective in promoting resistance to a wide array of bacterial and parasitic pathogens [14•, 57, 58], but limited information is available on fungi. During macrophage invasion by *C. neoformans*, NRAMP1 improved macrophage anti-cryptococcal activity at early, but not late stages of fungal invasion [59].

Neutrophils can also restrict Mn from pathogens using calprotectin (Cp). Calprotectin is highly expressed in neutrophils, making up about 40% of the total cytoplasmic protein content in these immune cells [60]. The metal binding properties of Cp are well defined, and this protein is widely recognized for its ability to restrict microbial access to Mn, Zn, Cu, and Fe [61–64]. Cp can withhold multiple metals from fungi in culture, including Mn, Zn, and Cu, and in animals, Cp is released in abundance at tissue sites of infection with *C. albicans* and *A. fumigatus* [64–66]. Cp-deficient mice display an increased fungal burden during *A. fumigatus* corneal infection [66] and also show an impaired ability to starve *C. albicans* for Zn during disseminated candidiasis [64]. When *C. albicans* invades the kidney, fungal mRNA markers for Zn starvation are strongly upregulated in WT mice, but not in Cp-deficient mice [64]. A similar effect of Cp on fungal Mn in vivo could not be examined due to the lack of any known fungal mRNA markers for Mn deficiency. Based on the strong effects of Cp on fungal Mn in vitro [64–66], this host protein is likely to restrict Mn from the pathogen in vivo as well.

In addition to these macrophage and neutrophil effects on Mn, recent studies have demonstrated whole tissue restriction of Mn during fungal invasion. Specifically, in a mouse model for disseminated candidiasis where the kidney is the major target tissue, whole kidney Mn levels decline during *C. albicans* invasion of the tissue [43••]. The liver is a secondary site of *C. albicans* infection, and like the kidney, liver Mn declines during fungal invasion [67••]. The mechanism for the loss in tissue Mn during fungal infection is not known. In the kidney, Mn levels are controlled by several Zn/Mn transporters including ZIP8. Interestingly, mutations in ZIP8 have been associated with higher susceptibility to inflammatory bowel disease and to infection by *Streptococcus pneumoniae* [68•, 69]. Future studies are warranted to address the possible role of ZIP8 or other host metal transporters in Mn limitation in the kidney during fungal invasion.

Conclusion

Like other eukaryotes, fungi rely on Mn to activate numerous enzymes including superoxide dismutases and glycosyltransferases. What sets fungi apart is their dependence on this metal for the formation of the fungal cell wall, particularly the outer mannose-containing layer that contributes to cell shape and integrity and modulates fungal recognition by the host immune system. With the importance of the cell wall in fungal growth and survival, it is not surprising that Mn deficiencies have been associated with defects in fungal morphogenesis and pathogenesis. In addition to cell wall effects, Mn has a key role as an antioxidant, which can be of particular importance to pathogenic fungi attacked by ROS from host immune cells. Mn is a co-factor for SOD enzymes, and in *C. albicans*, Mn drives maturation (through mannosylation) of extracellular Cu-SODs that detoxify host ROS [43••, 70]. Mn is also necessary for TOR nutrient signaling [13••], a process important for fungal morphogenesis [71]. Because fungi rely on Mn for such diverse processes, host withholding of this metal may be an effective antifungal tactic. Several immune responses are consistent with nutritional immunity for Mn. Macrophages can withhold Mn (and Fe) from fungi in the phagolysosome [59], and neutrophils release abundant metal-binding calprotectin at sites of infection [64–66]. Moreover, total kidney and liver Mn drop in response to tissue invasion by *C. albicans* [43••, 67••].

While a role for Mn in fungal pathogenesis is now established, there are still many unknowns. For example, how do pathogenic fungi control Mn homeostasis? Transporters for Zn, Fe, and Cu in pathogenic fungi are regulated at the mRNA level by transcription factors that sense these metals [3–5], but there is no known fungal transcription factor that senses and responds to Mn. Based on the Irving-Williams series [72], Mn is predicted to have a relatively low affinity for biological ligands compared to other metals,

and a Mn-specific trans-regulator may not be thermodynamically feasible. Rather than transcription, Mn transport in pathogenic fungi may be regulated in the same fashion as described for *S. cerevisiae*, namely by post-translational control of NRAMP transporter protein localization and turnover [16, 17, 19, 22–26]. Future studies are needed to understand how pathogenic fungi adapt to changes in environmental Mn, whether this occurs by regulating Mn transport and trafficking or by modulating the usage of Mn as enzymatic co-factor. There is also still much to be learned on how the host controls Mn availability to the fungus during infection. What is the mechanism underlying the total drop in kidney and liver Mn when *C. albicans* invades these tissues? With the availability of mouse mutants for Mn transport, an understanding may be imminent.

Lastly, the role of Mn in fungal infections is expected to have important implications regarding the increasing rise in fungal infections. Recent estimations show human fungal infections now affecting ~ 300 million people annually, with over 1 million deaths worldwide [73]. Fungal infections can be challenging to diagnose, and with a rising incidence of drug resistance, many fungal infections are difficult to treat [74]. Of particular concern is the recent emergence of new fungal pathogens, such as *Cryptococcus gattii* and the multidrug-resistant *Candida auris* [75–77]. Since fungi are also pathogens for non-human animals and plants, fungal infections are greatly impactful in agriculture and the ecosystem [78, 79]. There is clearly an urgent need for an improved understanding of fungal disease and for the development of novel therapeutic avenues. Could the fungal reliance on Mn represent an Achilles' heel worth exploring? Future studies should shed light on ways to exploit fungal dependence on Mn and other metals in tackling infectious fungi.

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Data Availability No datasets were generated or analyzed during the current study.

Declarations

Conflict of Interest The authors declare that they have no conflicts of interest.

Human and Animal Rights and Informed Consent All studies performed by the authors that were reported to involve animals have been published and were compliant with Johns Hopkins Institutional Animal Care and Use Committee and with the guidelines of the Animal Welfare Act and Public Health Service Policy.

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- • Of importance
- •• Of major importance

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