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MICROREVIEW

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An alloy of zinc and innate immunity: Galvanising host defence against infection

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Abstract

Innate immune cells such as macrophages and neutrophils initiate protective inflammatory responses and engage antimicrobial responses to provide frontline defence against invading pathogens. These cells can both restrict the availability of certain transition metals that are essential for microbial growth and direct toxic concentrations of metals towards pathogens as antimicrobial responses. Zinc is important for the structure and function of many proteins, however excess zinc can be cytotoxic. In recent years, several studies have revealed that innate immune cells can deliver toxic concentrations of zinc to intracellular pathogens. In this review, we discuss the importance of zinc status during infectious disease and the evidence for zinc intoxication as an innate immune antimicrobial response. Evidence for pathogen subversion of this response is also examined. The likely mechanisms, including the involvement of specific zinc transporters that facilitate delivery of zinc by innate immune cells for metal ion poisoning of pathogens are also considered. Precise mechanisms by which excess levels of zinc can be toxic to microorganisms are then discussed, particularly in the context of synergy with other antimicrobial responses. Finally, we highlight key unanswered questions in this emerging field, which may offer new opportunities for exploiting innate immune responses for anti-infective development.

KEYWORDS

antimicrobial, bacterial pathogens, host, innate immunity, pathogen, zinc toxicity, zinc transporters

1 | INTRODUCTION

The innate immune system provides the first line of defence against infection. Innate immune cells, including monocytes, macrophages, dendritic cells and neutrophils, initiate both inflammatory and antimicrobial responses to clear pathogens. During infection, pathogens require essential nutrients, including transition metals, for growth and

survival. Innate immune cells thus restrict the availability of specific transition metals to limit microbial growth, in a process termed nutritional immunity (Hood & Skaar, 2012). Conversely, phagocytes can also traffic toxic concentrations of metal ions as an antimicrobial response against intracellular pathogens (Sheldon & Skaar, 2019).

Zinc is a key component of enzymes across all domains of life. This metal is essential for the structure and/or function of many proteins; for example, the catalytic activity of numerous enzymes (Andreini & Bertini, 2012). Zinc also has roles in several signal transduction pathways, including in inflammatory and immune signalling (Haase & Rink, 2007; Pyle et al., 2017; Yamasaki et al., 2007). Despite these essential roles, excessive zinc can be cytotoxic and can cause

Abbreviations: CDF, cation diffusion facilitator; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MT/Mt, metallothionein (HUMAN/murine); NO, nitric oxide; ROS, reactive oxygen species; SLC/Slc, solute carrier (HUMAN/Murine); SPI-1, Salmonella pathogenicity island-1; S, Typhimurium, Salmonella enterica serovar Typhimurium; UPEC, uropathogenic E. coli.

tissue damage, organ failure and death (Bennett et al., 1997; Lee & Koh, 2010). Therefore, zinc homeostasis needs to be tightly regulated (Blencowe & Morby, 2003; Eide, 2006). Innate immune cells can mobilise zinc during infection to achieve either zinc sequestration or to deliver toxic concentrations of zinc to intracellular pathogens (Lonergan & Skaar, 2019; Sheldon & Skaar, 2019). Zinc sequestration has previously been extensively reviewed (Haase, 2013; Hood & Skaar, 2012; Jordan, Wang, Capdevila, & Giedroc, 2020; Lonergan & Skaar, 2019; Sheldon & Skaar, 2019). This review will discuss and highlight key unanswered questions about the emerging role of zinc toxicity as an antimicrobial response deployed by the innate immune system during infection.

2 | ZINC DEFICIENCY AND SUPPLEMENTATION IN INFECTIOUS DISEASE

While zinc toxicity in humans is very rare, approximately 20% of the world population is estimated to be at risk of inadequate zinc intake (Wuehler, Peerson, & Brown, 2005). Zinc deficiency is of particular concern for susceptibility to infectious diseases, due to the established role of zinc in immune functions (Gammoh & Rink, 2017). Indeed, sepsis severity is associated with low plasma zinc concentrations in adults (Besecker et al., 2011; Hoeger et al., 2017). Low plasma zinc levels are also linked to immunosenescence, an age-related decline in immune function accompanied by recurring infections and chronic inflammation (Maywald & Rink, 2015; Rink, Cakman, & Kirchner, 1998). Consistent with this, long-term zinc supplementation reduced infection incidence in healthy elderly subjects (Prasad et al., 2007). Furthermore, zinc supplementation in humans improves outcomes for multiple types of infectious disease, such as respiratory tract infections (Brooks et al., 2005; Sazawal et al., 1998), pneumonia (Bhutta et al., 1999; Valavi, Hakimzadeh, Shamsizadeh, Aminzadeh, & Alghasi, 2011), diarrhoeal disease (Ahmadipour, Mohsenzadeh, Alimadadi, Salehnia, & Fallahi, 2019; Al Tarique et al., 2010; Bhutta et al., 2000; Castillo-Duran, Vial, & Uauy, 1988; Sazawal et al., 1998) and viral infections (Matsumura et al., 2012; Varadinova et al., 1993). Of particular note, the WHO and UNICEF recommend zinc supplementation for the clinical management of acute diarrhoea in children, as a consequence of multiple studies demonstrating its efficacy in reducing both disease severity and duration (Penny, 2013).

In keeping with the clinical evidence above, the importance of zinc in host defence has been confirmed by modulating dietary intake of zinc in animal models of infection (summarised in Table S1). For example, zinc deficiency enhanced organ damage and mortality in a murine model of polymicrobial sepsis, with these effects being rescued by zinc supplementation (Knoell et al., 2009). Furthermore, zinc restriction in mice enhanced *Streptococcus pneumoniae* infection, likely due to impaired pathogen clearance by phagocytes (Eijkelkamp et al., 2019). Such studies are consistent with zinc having important roles in facilitating innate immune cell functions during infection.

3 | EVIDENCE FOR ZINC TOXICITY AS AN INNATE IMMUNE ANTIMICROBIAL RESPONSE

3.1 | Mobilisation of zinc by innate immune cells during infection

Zinc is mobilised and concentrated in specific tissues during inflammation and infection. For example, infection of mice with *S. pneumoniae* resulted in increased zinc concentrations in the lungs, brain, nasopharynx and blood (McDevitt et al., 2011), with this pathogen subsequently shown to co-localise with zinc-enriched regions in murine lungs (Eijkelkamp et al., 2019). Several studies, utilising either fluorescent zinc-binding dyes or direct elemental analysis, have also documented fluctuations in zinc concentrations within immune cells in response to infection or microbial products (Table 1). Zinc accumulation in phagosomes during infection has been observed in multiple cellular systems. Macrophages mobilise and traffic zinc to phagosomes and intracellular compartments that co-localise with various bacterial pathogens, including *Mycobacterium tuberculosis*, *Salmonella enterica* serovar Typhimurium and *Escherichia coli* (Table 1). Subsequent studies have confirmed that zinc vesicles co-localised with an *E. coli* strain that specifically reports zinc stress (Stocks et al., 2019). Zinc accumulation in lysosomes and azurophilic granules has also been observed in neutrophils, implicating zinc delivery to phagocytosed *Streptococcus pyogenes* (Ong, Berking, Walker, & McEwan, 2018). Interestingly, in the phagocytic soil amoeba *Dictyostelium discoideum* that employs phagocytosis to feed on bacteria, zinc was also delivered towards *Mycobacterium smegmatis*-containing vacuoles (Barisch et al., 2018). Combined with evidence below linking such effects to zinc stress, these studies suggest innate immune cells can deliver zinc to poison intracellular bacteria.

3.2 | Pathogens resist zinc toxicity by detoxification during infection

Much of the evidence for innate immune zinc toxicity is based on the necessity of bacterial zinc export systems for survival within innate immune cells. As for mammals, bacteria maintain zinc homeostasis by expression of zinc importers and exporters. Zinc export is achieved via P-type ATPases including ZntA (Beard, Hashim, Membrillo-Hernández, Hughes, & Poole, 1997; Rensing, Mitra, & Rosen, 1997), as well as the putative zinc exporters CtpC and CtpG (Botella et al., 2011) (Figure 1). In addition, cation diffusion facilitators (CDF) including ZitB (Grass et al., 2001; Wang, Hosteen, & Fierke, 2012) and heavy metal efflux systems such as CzcCBA promote zinc efflux in bacteria (Blencowe & Morby, 2003; Figure 1). Another CDF, CzcD, also mediates zinc export in both Gram-negative bacteria such as *Acinetobacter baumannii* (Alquethamy et al., 2019) and Gram-positive bacteria such as *S. pyogenes* (Ong, Gillen, Walker, Barnett, & McEwan, 2014). CzcD performs this function via an antiport mechanism (Guffanti, Wei, Rood, & Krulwich, 2002). When zinc levels are

TABLE 1 Innate immune cells mobilise zinc in response to specific stimuli and/or infection

Species	Cell type	Stimulation	Evidence	References
Human	Monocyte-derived macrophages	Chronic NOD2 stimulation	↑ Intracellular Zn ²⁺ . Upregulated <i>MT</i> expression.	Lahiri and Abraham, (2014)
		<i>M. tuberculosis</i> infection	↑ Zn ²⁺ . Lysosomal/endosomal Zn ²⁺ accumulation.	Botella et al. (2011)
		<i>E. coli</i> infection	↑ Intracellular Zn ²⁺ . Zn ²⁺ co-localised with phagolysosomal <i>E. coli</i> . Zn ²⁺ co-localised with Zn ²⁺ -stressed <i>E. coli</i> . Zn ²⁺ vesicles co-localised with <i>E. coli</i> .	Botella et al. (2011) Stocks et al. (2019) Kapetanovic et al. (2016)
	THP-1	LPS	Vesicular Zn ²⁺ accumulation.	Kapetanovic et al. (2016)
		<i>S. Typhimurium</i> infection	Infection induced Zn ²⁺ vesicle formation. Zn ²⁺ co-localised with ΔSPI-1 mutant strain.	Kapetanovic et al. (2016)
		LPS	Vesicular Zn ²⁺ accumulation.	Kapetanovic et al. (2016)
Mouse	Peritoneal macrophages	TNF (pre/post infection)	↑ Zn ²⁺ in <i>M. avium</i> -containing vacuoles.	Wagner et al. (2005)
		IFNγ (post-infection)	↑ Zn ²⁺ in <i>M. avium</i> -containing vacuoles.	Wagner et al. (2005)
		<i>M. tuberculosis</i> / <i>M. smegmatis</i> infection	↑ Phagosomal Zn ²⁺ .	Wagner et al. (2005)
	Bone marrow-derived macrophages	IL-4	Mt-3 and Slc30a4-dependent ↓ of labile Zn ²⁺ stores.	Subramanian Vignesh et al. (2016))
		<i>E. coli</i> infection	Intramacrophage <i>E. coli</i> reported Zn ²⁺ stress.	Stocks et al. (2019))
		<i>C. glabrata</i> infection	Zn ²⁺ accumulation in fungal vacuole.	Riedelberger et al. (2020)
	RAW 264.7	<i>S. Typhimurium</i> infection	↑ Intracellular Zn ²⁺ .	Wu et al. (2017)
<i>C. glabrata</i> infection		Zn ²⁺ accumulation in fungal vacuole.	Riedelberger et al. (2020)	

elevated, the cytosolic regulators ZntR (Brocklehurst et al., 1999) and GcZA/SczA (Kloosterman, van der Kooi-Pol, Bijlsma, & Kuipers, 2007; Martin et al., 2017) induce *zntA* and *czcD* expression, respectively (Figure 1).

Several zinc exporters and detoxification systems are induced in various pathogens during infection of innate immune cells and/or in vivo models of infection (Table 2). For example, zinc export was upregulated by *S. pneumoniae* following infection of human neutrophils, the macrophage-like cell line THP-1, and within an intranasal murine infection model (Eijkelkamp et al., 2019). In addition, zinc efflux systems were upregulated by *M. tuberculosis* (Botella et al., 2011), *S. Typhimurium* (Kapetanovic et al., 2016) and *E. coli* (Stocks et al., 2019) upon infection of macrophages. Several studies have also highlighted the necessity of pathogen zinc export systems for their survival during infection (Table 2). Human macrophages were able to more efficiently clear *M. tuberculosis* and *E. coli* that lack zinc export systems by comparison to isogenic wild-type strains (Botella et al., 2011; Stocks et al., 2019). Additionally, zinc-sensitive mutants of *S. pyogenes* (Ong et al., 2014; Ong et al., 2018), *S. pneumoniae* (Eijkelkamp et al., 2019) and *E. coli* (Kapetanovic et al., 2016) were more susceptible to neutrophil killing. Similarly, *S. pneumoniae*, *S. pyogenes* and *E. coli* which lack regulators required for zinc exporter expression exhibited reduced intracellular survival in THP-1 cells (Martin et al., 2017), neutrophils (Ong et al., 2014) and macrophages (Stocks et al., 2019), respectively. Recent evidence suggests that the innate immune zinc toxicity response is not just limited to bacterial

infection. In murine macrophages, the fungal pathogen *Candida glabrata* required the zinc transporter *ZRC1* for survival (Riedelberger et al., 2020). This study implicated a role for *ZRC1* in the sequestration of excess zinc into intracellular vacuoles during infection. Together, this literature implies that a variety of pathogens are subjected to zinc toxicity during infection of innate immune cells and employ zinc detoxification systems to resist this response.

3.3 | Pathogen evasion of zinc toxicity responses during infection

Pathogens require strategies to resist or evade host innate immune responses to enable survival during infection. In addition to resistance by zinc detoxification systems (described above), some pathogens can also evade macrophage zinc-toxicity responses. Moreover, *Salmonella* actually employs both resistance and evasion to subvert zinc toxicity responses deployed by macrophages. While wild-type *S. Typhimurium* did not co-localise with zinc vesicles in primary human macrophages, a *Salmonella* pathogenicity island-1 (ΔSPI-1) mutant strain co-localised with zinc vesicles, upregulated *zntA* and was compromised for intramacrophage survival (Kapetanovic et al., 2016). However, a *S. Typhimurium* ΔSPI-1/Δ*zntA* mutant was no more sensitive to killing by human macrophages than an isogenic ΔSPI-1 mutant strain, suggesting that *Salmonella* also has SPI-1- and ZntA-independent mechanisms to resist macrophage-mediated zinc toxicity (Kapetanovic

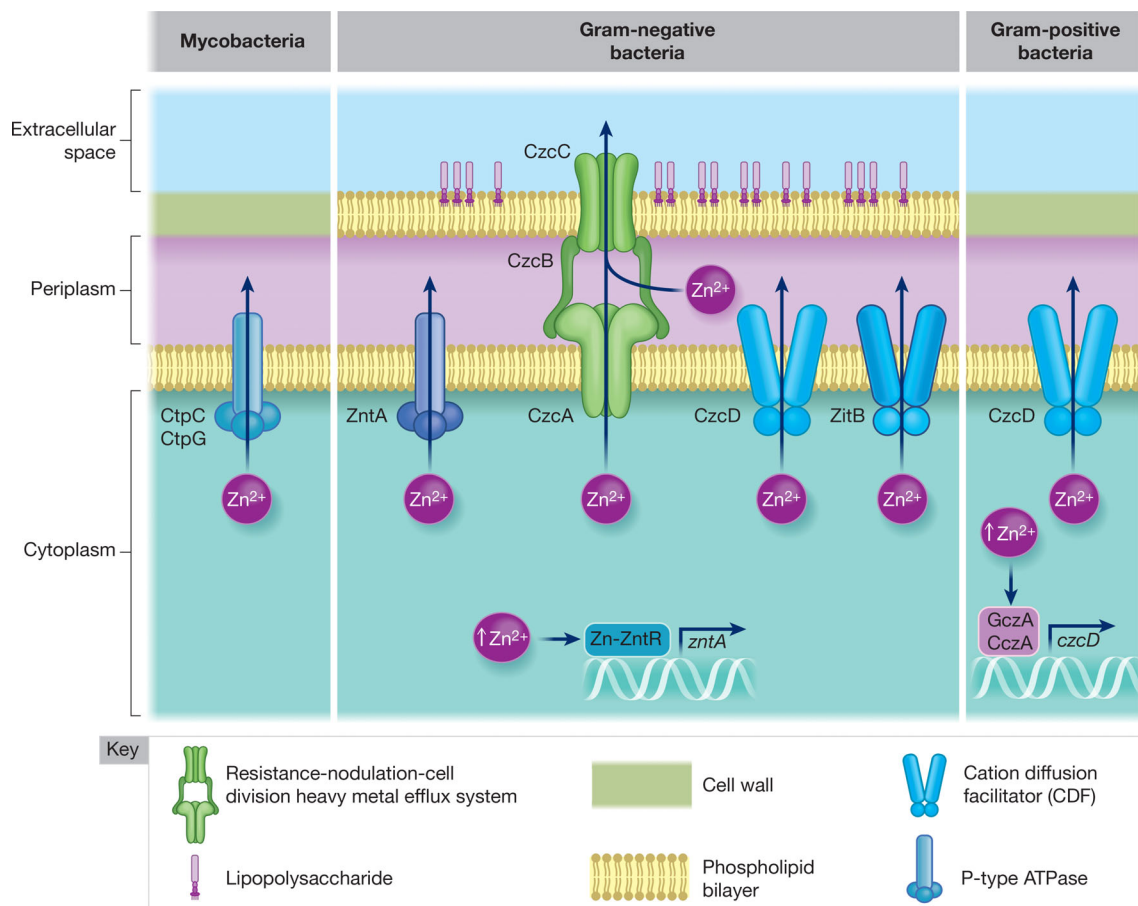


FIGURE 1 Bacterial zinc efflux systems. Bacteria express various exporters which transport zinc from the cytosol to maintain homeostasis in conditions of high zinc availability. Expression of some exporters are regulated by zinc-sensing transcription factors, thus enabling their selective induction under conditions of zinc stress

et al., 2016). Other bacterial pathogens such as uropathogenic *E. coli* (UPEC) also evade the macrophage zinc toxicity response. In contrast to non-pathogenic *E. coli*, the majority of intramacrophage UPEC did not report zinc stress, as indicated by a novel zinc-stress reporter system based on zinc-inducible *zntA* expression (Stocks et al., 2019). Consistent with this, intramacrophage survival of zinc-sensitive *zntA*- and *zntR*-deficient UPEC mutants was not reduced, in contrast to observations with corresponding mutants of a non-pathogenic *E. coli* strain (Stocks et al., 2019). This suggests that UPEC effectively evades macrophage-mediated zinc toxicity. What remains to be determined are the specific zinc toxicity evasion strategies employed by *S. Typhimurium* and UPEC.

4 | HOST CELL MECHANISMS UNDERPINNING ANTIMICROBIAL ZINC DELIVERY

Innate immune delivery of zinc for microbial intoxication is likely to be driven by multiple, interconnected mechanisms, including zinc import, zinc export and interactions with metallothioneins (MTs; Figure 2).

Mammalian cells utilise 14 highly conserved solute carrier (SLC) 39A family members [also referred to as Zrt-, Irt-like proteins (ZIP)] for the import of zinc and/or other metal ions from the extracellular space and the lumen of organelles into the cytoplasm (Eide, 2006; Hara et al., 2017). In addition, mammals possess 10 CDF zinc exporters of the SLC30A family (also referred to as ZnT) that can export zinc and/or other metal ions from the cytoplasm into luminal compartments or the extracellular space (Eide, 2006; Huang & Tepasamorndech, 2013). It is likely that both SLC39A and SLC30A zinc transporters are involved in the coordinated shuttling of zinc pools towards engulfed bacteria. In this manner, zinc importers may take up zinc from the extracellular environment and/or cellular compartments to maintain adequate cytosol zinc concentrations, while zinc exporters may concentrate zinc ions into vesicles or bacteria-containing compartments.

Various innate immune stimuli induce zinc mobilisation in macrophages. For example, agonists of several Toll-like receptors (TLRs) increased available zinc as determined by intracellular staining with FluoZin-3 in RAW 264.7 cells and human monocytes (Brieger, Rink, & Haase, 2013). In addition, TLR 3, 4 and 7 stimulation promoted vesicular zinc accumulation in primary human macrophages and THP-1

TABLE 2 Evidence for innate immune-mediated zinc toxicity against intracellular pathogens

Pathogen	Infection model	Phenotype	References
<i>Zinc transporters</i>			
<i>M. tuberculosis</i>	Human macrophage	↑ <i>ctpC</i> , <i>ctpG</i> , <i>ctpV</i> expression. Growth defect in Δ <i>ctpC</i> mutant.	Botella et al. (2011)
<i>S. Typhimurium</i>	Mouse infection model (intraperitoneal)	Δ <i>zntA</i> Δ <i>zitB</i> strain out-competed by wild-type strain in liver and spleen.	Frawley et al. (2018)
	Human macrophage	↑ <i>zntA</i> expression and ↑ susceptibility to killing in Δ SPI-1 mutant strain. SPI-1 prevents Zn ²⁺ vesicle co-localisation with bacteria.	Kapetanovic et al. (2016)
<i>E. coli</i>	Human neutrophil	Δ <i>zntA</i> mutant strain more susceptible to killing.	Kapetanovic et al. (2016)
	Human macrophage	↑ <i>zntA</i> expression. ↓ Survival Δ <i>zntA</i> mutant strain.	Kapetanovic et al. (2016) Botella et al. (2011)
	Murine macrophage	↑ <i>zntA</i> expression. ↓ survival Δ <i>zntA</i> mutant strain. Intramacrophage <i>E. coli</i> reported Zn ²⁺ stress (<i>zntA</i> -driven fluorescence).	Stocks et al. (2019)
		Intramacrophage <i>E. coli</i> reported Zn ²⁺ stress (<i>zntA</i> -driven fluorescence).	Stocks et al. (2019)
Mouse infection model (intraperitoneal)	↓ Dissemination of Δ <i>zntA</i> mutant strain in liver and spleen.	Stocks et al. (2019)	
<i>S. pneumoniae</i>	Human neutrophil	↑ <i>czcD</i> and ↓ <i>phtE</i> (Zn ²⁺ importer) expression. Zn ²⁺ supplementation ↓ wild-type and Δ <i>czcD</i> mutant survival.	Eijkelkamp et al. (2019)
	Mouse infection model (intranasal)	Zn ²⁺ co-localised with bacteria in lung cavity. ↑ <i>czcD</i> and ↓ <i>phtE</i> expression. ↓ Δ <i>czcD</i> mutant abundance in lungs.	Eijkelkamp et al. (2019)
	THP-1	↑ <i>czcD</i> expression. Zn ²⁺ supplementation ↓ wild-type and Δ <i>czcD</i> mutant survival.	Eijkelkamp et al. (2019)
<i>S. pyogenes</i>	Human neutrophil	Δ <i>czcD</i> mutant more susceptible to killing.	Ong et al. (2014); Ong et al. (2018)
	Mouse infection model (invasive subcutaneous)	Δ <i>czcD</i> mutants ↓ mortality of mice. Δ <i>czcD</i> mutants had ↓ survival relative to wild-type during competitive infection.	Ong et al. (2014)
	Mouse infection model (invasive subcutaneous)	Δ <i>czcD</i> mutants ↓ initial lesion size, dissemination in blood and mortality of mice.	Ong et al. (2018)
<i>C. glabrata</i>	Murine macrophage	Survival defect in Δ ZRC1 mutants.	Riedelberger et al. (2020)
<i>Pseudomonas aeruginosa</i>	THP-1	↑ <i>cadA</i> and <i>czcC</i> expression during infection.	Ducret, Gonzalez, Leoni, Valentini, and Perron (2020)
<i>Regulators of zinc exporters</i>			
<i>E. coli</i>	Human macrophage	↓ Survival Δ <i>zntR</i> mutant strain.	Stocks et al. (2019)
<i>S. pneumoniae</i>	THP-1	↓ Survival of Δ <i>sczA</i> mutant.	Martin et al. (2017)
<i>S. pyogenes</i>	Human neutrophil	Δ <i>gczA</i> mutant more susceptible to killing.	Ong et al. (2014)
	Mouse infection model (subcutaneous)	↓ Virulence and ↓ survival of Δ <i>gczA</i> mutants relative to wild-type strains.	Ong et al. (2014)

cells that had been differentiated into macrophage-like cells (Kapetanovic et al., 2016). As TLRs initiate late-stage antimicrobial responses (Stocks, Schembri, Sweet, & Kapetanovic, 2018), zinc toxicity may be engaged by innate immune cells to defend against persistent intracellular pathogens. It is therefore likely that these receptors, and potentially other receptors that sense infections and/or danger, regulate the expression and/or functions of SLC39A, SLC30A and/or

MT family members to initiate and/or sustain the zinc toxicity response (Figure 2).

Although it is currently unknown if specific importers are involved in zinc-mediated antimicrobial responses, roles for some SLC39A transporters appear likely. For example, SLC39A8 is a manganese and zinc importer (Begum et al., 2002; Lin et al., 2017) that is induced by LPS in monocytes (Pyle et al., 2017) and macrophages (Liu

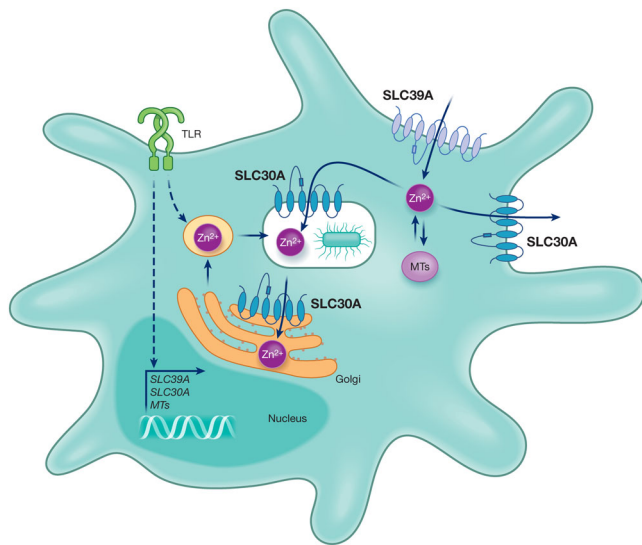


FIGURE 2 Innate immune cells mobilise zinc during infection. Innate immune cells traffic zinc towards pathogen-containing intracellular compartments, likely through the coordinated actions of zinc importers, zinc exporters and metallothioneins (MTs). SLC39A family members import zinc from extracellular environments into the cytoplasm, while members of the SLC30A family export zinc from the cytoplasm for its delivery to pathogens and/or to maintain zinc homeostasis. MTs regulate cytosolic zinc homeostasis and may control the delivery of zinc to zinc transporters in response to infection. Innate immune signalling systems, including Toll-like receptors, induce the expression of various zinc transporters and MTs, as well as zinc vesicle accumulation in immune cells

et al., 2013), with defined roles in regulating inflammatory responses (Hall, Smith, Katafiasz, Bailey, & Knoell, 2019; Liu et al., 2013; Pyle et al., 2017). *SLC39A14*, which is closely related to *SLC39A8* (Girijashanker et al., 2008), is also LPS-inducible and regulates cytokine production in human macrophages (Sayadi, Nguyen, Bard, & Bard-Chapeau, 2013). As *SLC39A8* and *SLC39A14* are both induced by LPS in macrophages, these transporters may be involved in not only inflammatory responses, but also LPS-induced zinc mobilisation for metal ion poisoning. This possibility remains to be investigated.

With respect to SLC30A family members, *SLC30A1* expression is induced by LPS in primary human macrophages, with its ectopic expression in monocytic THP-1 cells being sufficient to drive the formation of zinc vesicles and to subject intracellular *E. coli* to zinc stress (Stocks et al., 2020). Moreover, this study demonstrated that *SLC30A1* localised to compartments containing zinc-stressed *E. coli*. However, silencing of *SLC30A1* in primary human macrophages did not affect bacterial clearance, suggesting that other zinc exporters may also contribute to this response. One candidate that may play a role in the absence of *SLC30A1* is *SLC30A4*. Interleukin (IL)-4 treatment upregulated *Slc30a4* in murine macrophages, resulting in an increase in labile zinc within the phagolysosome (Subramanian Vignesh et al., 2016). *Slc30a4*, along with *Slc30a7*, was also implicated in granulocyte macrophage colony stimulating factor-mediated zinc sequestration in the Golgi in murine macrophages (Subramanian

Vignesh, Landero Figueroa, Porollo, Caruso, & Deepe, 2013). This sequestration was associated with a zinc starvation response against an intramacrophage fungal pathogen, but it is possible that these transporters may additionally be involved in the delivery of zinc toxicity to different pathogens and/or in different microenvironments. Consistent with a role for one or more SLC30A family members in the zinc toxicity response in human and mouse macrophages, zinc was rapidly delivered towards *M. smegmatis*-containing vacuoles via SLC30A homologues in *D. discoideum* (Barisch et al., 2018). The predicted mechanism of action of SLC30A family zinc transporters has been modelled using the *E. coli* CDF proteins ZitB (Chao & Fu, 2004) and YiiP (Lu & Fu, 2007), which export zinc via a H^+ antiport mechanism (Chao & Fu, 2004; Grass et al., 2005). Indeed, *SLC30A1* homodimers utilise a H^+ electrochemical gradient to drive Zn^{2+} efflux in exchange for H^+ in a pH-driven, sodium-independent and calcium-sensitive manner (Shusterman et al., 2014). Thus, the acidic environment of phagolysosomes might enable the delivery of zinc to pathogens by one or more SLC30A transporters.

Another important factor influencing zinc transport in mammalian cells is control by the MT family. MTs are a family of monomeric metal-binding proteins that regulate cytosolic zinc homeostasis under both steady and non-steady state conditions (Colvin, Holmes, Fontaine, & Maret, 2010). The MTs are encoded by 10–12 genes (chromosome 16) and 4 genes (chromosome 8) in humans and mice, respectively (Cox & Palmiter, 1983; Vařák & Meloni, 2011). Some studies have demonstrated that MTs are regulated in innate immune cells during infection (Botella et al., 2011; Subramanian Vignesh et al., 2013). Moreover, silencing of the transcription factor metal-regulatory factor-1, which controls cellular responses to metals, disrupted MT expression, prevented intracellular zinc accumulation and impaired bacterial clearance in human macrophages chronically stimulated with the microbial product muramyl dipeptide (Lahiri & Abraham, 2014), thus implicating MTs in antimicrobial responses. More recently, *Mt-1* and *Mt-2* were shown to be required for zinc intoxication against phagocytosed *C. glabrata* in murine macrophages (Riedelberger et al., 2020). This study demonstrated that Mts did not co-localise with the pathogen during infection, suggesting that Mts are involved in zinc mobilisation rather than direct delivery of zinc to intracellular pathogens. It is likely that MTs cooperate with zinc transporters, providing a sophisticated network of effector proteins and zinc chaperones to control the distribution of intracellular metal ions for effective inflammatory and antimicrobial responses.

5 | ZINC AS AN ANTIMICROBIAL EFFECTOR MOLECULE

5.1 | Direct antimicrobial impacts of zinc

High concentrations of zinc are likely toxic to pathogens through multiple mechanisms. Zinc is predicted to preferentially bind to proteins in place of other first-row transition metal ions, resulting in mismetallation of essential proteins (Braymer & Giedroc, 2014; Stafford et al., 2013; Xu &

lmlay, 2012). For example, zinc irreversibly binds to manganese-acquisition proteins in *S. pneumoniae* (Couñago et al., 2014; McDevitt et al., 2011), *Bacillus anthracis* (Vigonsky et al., 2015) and *Staphylococcus pseudintermedius* (Abate et al., 2014). Thus, zinc excess can induce manganese deficiency in bacteria, leading to increased sensitivity to oxidative stress (Eijkelkamp et al., 2014; McDevitt et al., 2011). This concept has been recently confirmed in a study whereby ionophores that induced zinc accumulation in *Streptococcus uberis* disabled oxidative stress protection through inhibition of manganese-dependent superoxide dismutase (Harbison-Price et al., 2020). Zinc excess can also dysregulate homeostasis of other trace metal ions. Excessive zinc can induce copper depletion, which may be driven by dysregulation of copper transporters and detoxification systems (Hassan et al., 2017; Xu et al., 2019). In addition, zinc excess increases the concentration of iron in *E. coli* by transiently inducing expression of iron-uptake genes and downregulating iron storage genes (Xu et al., 2019). Zinc may also have direct impacts on key cellular processes required for microbial survival. For example, excessive intracellular zinc can disrupt iron-sulfur (4Fe-S) complex biogenesis in *E. coli*, thereby arresting key metabolic and growth pathways (Li et al., 2019; Xu & lmlay, 2012). In addition, excess zinc impaired capsule synthesis and perturbed glycolysis through inhibition of glycolytic enzymes in *S. pyogenes* (Ong, Walker, & McEwan, 2015). Thus, high concentrations of zinc can dysregulate many facets of homeostasis in microorganisms to exert its antimicrobial effects.

5.2 | Cooperation between zinc and other innate immune antimicrobial pathways

In addition to direct toxicity, zinc may also promote bacterial clearance through indirect mechanisms, particularly through enhancement of, or synergism with, other innate immune antimicrobial pathways. For example, basal and inducible autophagy is dependent on zinc in various cell types (Hung, Huang, & Pan, 2013; Hwang et al., 2010; Lee & Koh, 2010; Liuzzi & Yoo, 2013). In human macrophages, signalling via the pattern recognition receptor NOD2 increased intracellular zinc levels to promote autophagy and intracellular clearance of *S. Typhimurium*, *S. aureus* and adherent invasive *E. coli* (Lahiri & Abraham, 2014). Therefore, regulated zinc trafficking in innate immune cells may contribute to autophagy-mediated control of intracellular pathogens.

Innate immune cells use reactive oxygen species (ROS), generated either through the NADPH oxidase system or through mitochondrial activity, as an antimicrobial weapon. Some evidence suggests that zinc may act cooperatively with ROS to exert antimicrobial effects. The growth of zinc-sensitive *E. coli* mutants was synergistically inhibited when bacteria were cultured in the presence of both zinc and the superoxide anion generator paraquat (Stocks et al., 2019). In contrast, zinc chelation by MTs was required for robust ROS generation upon challenge with *C. glabrata* (Riedelberger et al., 2020), *S. Typhimurium* (Wu et al., 2017) and *Histoplasma capsulatum* (Subramanian Vignesh et al., 2013) in murine or human macrophages. Therefore, the precise interplay between zinc and ROS during antimicrobial responses is likely to be context dependent.

In addition to ROS, innate immune cells also generate nitric oxide (NO) for free radical attack of intracellular pathogens. There is some evidence that zinc may influence NO production and/or that NO itself can initiate zinc toxicity. Zinc chelation abrogated TLR4- and TLR3-inducible nitric oxide synthase (iNOS/Nos2) expression that is required for inducible NO production in murine macrophages (Brieger et al., 2013). In bacteria, nitrosative stress may also interfere with zinc homeostasis. In *Salmonella*, NO triggers zinc release from multiple metalloproteins, including those involved in nitrosative stress sensing (Henard et al., 2014), cell metabolism, protein synthesis (Frawley et al., 2018) and DNA binding (Schapiro, Libby, & Fang, 2003). In addition, pharmacological inhibition of Nos2 in an intraperitoneal murine infection model restored bacterial loads of a *S. Typhimurium* $\Delta zntA\Delta zitB$ mutant to those of wild-type levels in murine spleen and liver, suggesting that NO may induce zinc toxicity in *Salmonella* (Frawley et al., 2018). This suggests that, under nitrosative stress, effective zinc efflux is required to maintain homeostasis and survival of *Salmonella* during murine infection (Frawley et al., 2018). Of note, however, TLR signalling induces the zinc toxicity response (Kapetanovic et al., 2016), but not NOS2 expression (Gross et al., 2014), in human macrophages. Thus, it seems unlikely that NO production contributes to the susceptibility of zinc-sensitive bacterial mutants in these cells.

Zinc toxicity may also be influenced by other metal ions during antimicrobial responses. Copper is utilised by the innate immune system as an antimicrobial weapon against various pathogens (Sheldon & Skaar, 2019). Non-toxic copper concentrations enhanced the cytotoxic effects of zinc on both *S. Typhimurium* and *E. coli* $\Delta zntA$ mutants, with these effects being rescued by *zntA* complementation (Kapetanovic et al., 2016). Similarly, co-treatment of *A. baumannii* with zinc and copper resulted in altered membrane composition and increased susceptibility to ROS (Hassan et al., 2017). Together, these studies suggest that copper and zinc may act cooperatively to exert an antimicrobial effect. Indeed, the concentrations of both zinc and copper were increased in Mycobacterium-containing phagosomes of murine macrophages under certain conditions (Wagner et al., 2005). In keeping with this concept, in LPS-stimulated human macrophages, vesicular zinc and copper partially co-localised (Kapetanovic et al., 2016). This raises the possibility of spatial and/or time-dependent cooperativity of these two metal ions, supporting the 'brass dagger' model of host defence (German, Doyscher, & Rensing, 2013). However, co-supplementation of THP-1 cells with zinc and copper did not affect clearance of *A. baumannii* (Hassan et al., 2017), suggesting that other exogenous factors and/or cell types may be important for combinatorial antimicrobial effects of these two metal ions.

6 | FINAL REMARKS AND FUTURE DIRECTIONS

In conclusion, zinc toxicity is emerging as an antimicrobial mechanism employed by innate immune cells to combat many

microorganisms, particularly bacterial and fungal pathogens. Significant advances have been made in our understanding of immune cell types that utilise zinc toxicity as an antimicrobial response, the pathogens which are subjected to zinc stress during infection, and the innate stimuli that drive this antimicrobial response. However, as highlighted throughout this review, key questions still remain. Specifically, the molecular mechanisms by which zinc is mobilised towards engulfed pathogens, the precise evasion mechanisms employed by pathogens, and the contributions of other antimicrobial pathways to zinc toxicity require further elucidation. Intriguing recent evidence also points to intertwining effects of the seemingly oppositional antimicrobial pathways of zinc sequestration and zinc toxicity. In a murine infection model, *A. baumannii* was subjected to zinc starvation in the respiratory tract, but experienced zinc intoxication in the spleen (Alquethamy et al., 2019). Similarly, another study showed that while neutrophils employ zinc sequestration against extracellular *S. pyogenes*, phagocytosed bacteria were subjected to zinc toxicity (Ong et al., 2018). One possibility is that the capacity of concentrated zinc to be cytotoxic to intracellular pathogens is enhanced by initial zinc starvation encountered in extracellular environments and/or in different tissues. In this way, innate immune-mediated zinc starvation may predispose microorganisms to subsequent zinc poisoning.

As increasing rates of antimicrobial resistance threaten global health worldwide, there is an urgent need for the development of novel therapies for infectious diseases. One approach under investigation is harnessing of innate immune antimicrobial responses through 'host-directed therapies'. Enhancing our understanding of the molecular mechanisms by which zinc is delivered to engulfed pathogens for metal ion poisoning, as well as the subversion mechanisms employed by pathogens to resist and/or evade zinc toxicity, could ultimately guide the development of new anti-infectives. Of note, zinc ionophores induce intracellular zinc accumulation, with one such ionophore recently passing second phase human clinical trials for the treatment of Alzheimer's disease and Huntington's disease (Huntington Study Group Reach2HD Investigators, 2015; Lannfelt et al., 2008; Villemagne et al., 2017). These zinc ionophores also have antibacterial activity against a variety of Gram-positive bacterial pathogens and can act synergistically with antibiotics (Bohlmann et al., 2018; Harbison-Price et al., 2020). Ionophores have also been effective in reversing antibiotic resistance in Gram-negative bacteria (Jen et al., 2020). Therefore, it may also be possible to mimic innate immune-mediated zinc toxicity through application of zinc ionophores. Therapeutics that target zinc homeostasis, either through host or pathogen manipulation, may thus provide new opportunities for combating antibiotic-resistant bacterial infections. Moreover, knowledge of how zinc toxicity responses are engaged by the host and subverted by pathogens will likely deliver fascinating new insights into the host-pathogen dynamic.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Jessica B. von Pein, Matthew J. Sweet, Claudia J. Stocks, Ronan Kapetanovic, Mark A. Schembri: Wrote the manuscript, after Jessica B. von Pein generated the initial draft. Jessica B. von Pein: Created the figures. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed in this review. This review was composed entirely from previously published data.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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