Bacterial killing in macrophages and amoeba: do they all use a brass dagger?

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Macrophages are immune cells that are known to engulf pathogens and destroy them by employing several mechanisms, including oxidative burst, induction of Fe(II) and Mn(II) efflux, and through elevation of Cu(I) and Zn(II) concentrations in the phagosome (`brass dagger'). The importance of the latter mechanism is supported by the presence of multiple counteracting efflux systems in bacteria, responsible for the efflux of toxic metals. We hypothesize that similar bacteria-killing mechanisms are found in predatory protozoa/amoeba species. Here, we present a brief summary of soft metal-related mechanisms used by macrophages, and perhaps amoeba, to inactivate and destroy bacteria. Based on this, we think it is likely that copper resistance is also selected for by protozoan grazing in the environment.

Precipitation of ferric iron and the consequent appearance of banded iron formations, which stopped approximately 1.8 billion years ago, are evidence of slow changes in the ocean environment caused by cyanobacteria producing oxygen in significant amounts [1]. As oxygen levels rose, microorganisms had to deal with reactive oxygen species and with the much greater concentrations of soft metals that became available in soluble form through oxidation of sulfide minerals. Consequently, organisms started using soft metals, such as zinc and copper, in their enzymes [2]. Since zinc is a borderline soft metal, concentrations of bioavailable zinc were probably higher than stronger soft metals, such as copper or cadmium. At the same time, intracellular levels of these metals had to be carefully regulated since soft metals, such as Cu(I), Zn(II) and Cd(II), can damage exposed FeS clusters in enzymes needed for normal cell functioning [3]. Importantly, it was around in the time period from approximately 3.1 to 2 billion years ago that eukaryotic cells with organelles and nuclei started to appear.

It is thought that the first eukaryotic cell was a phagotrophic heterotroph with cilium. How did these amoeba-like cells kill their prey? Although there is no certain answer to date, one can assume that killing mechanisms prior to oxygenation must have been very different from today owing to lack of available soft metals and the inability to form reactive oxygen species. Most research regarding the killing of bacteria has been conducted with contemporary macrophages as they are an integral part of innate immunity. Macrophages engulf invading bacteria and fungi and then kill them in the phagosome. How exactly this is achieved is still a matter of debate, as undoubtedly it is not only a single mechanism [4]. Certainly, the mere presence of digestive enzymes would not be able to kill most microbes. An additional effect is achieved by the action of the vacuolar H^+–ATPase, causing acidification of the phagosomal milieu. An acidic environment would not only make life unpleasant and increase protease activity, but also greatly enhance solubility of Cu(I) and, together with Cl^-, prevent disproportionation of Cu(I) into Cu(II) and Cu(0). This, in effect, would greatly increase Cu(I) toxicity. Reactive oxygen and reactive nitrogen species have both been shown to have a role in the killing of microbes [5]. Superoxide and peroxide with chloride can create hypochlorous acid, which is known to kill microbes. In addition, peroxide together with Fe(II) or Cu(I) can generate hydroxyl ion, which is known to possess antimicrobial activity [6]. One of the defense lines against bacterial invasion in macrophages is accumulation of Cu(I) and Zn(II) in the phagosome [7–9]. Their presence, in combination with reactive oxygen species, causes disruption of the energy cycle in bacteria through degradation of FeS clusters [3,10,11]. At the same time, the much needed cations for bacterial survival Fe(II) and Mn(II) are withdrawn from the phagosome by Nramp1 [12,13], calprotectin [14] and also by ferroportin, which is a copper-induced efflux system for

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ferrous iron (and probably Mn[II]) [15,16]. Therefore, soft metal poisoning goes after the Achilles' heel of every cell: a reducing cytoplasm with the cellular metabolism dependent on FeS clusters (Figure 1). Damage to FeS clusters can only be repaired by systems, such as iron-sulfur cluster or sulfur mobilization, requiring both iron and sulfur in the form of cysteine. In fact, *Escherichia coli* induction of the OxyR-regulated *suf* operon, encoding the much more stress-resistant sulfur mobilization system for FeS assembly, is required, whereas the iron-sulfur cluster system itself is not functional under high H_2O_2 stress [11,17]. Copper stress in *Bacillus subtilis* induces the expression of genes responsible for iron acquisition and FeS cluster assembly [18]. Concomitant compensatory mechanisms in bacteria include shifting from an iron-dependent metabolism towards a manganese-dependent metabolism, supported by an increase in expression of Mn uptake systems, such as MntH in *E. coli* or SitABCD in *Salmonella typhimurium* [19–21]. This change in metal utilization can be observed most dramatically in *Borrelia burgdorferi*, the causative agent of Lyme disease, where iron-containing enzymes were completely replaced by manganese-containing metalloenzymes [22,23]. Another indication of the importance of the FeS cluster containing enzymes is the inability of branched chain amino acid auxotrophic strains of *Mycobacterium tuberculosis* to grow in macrophages [24,25] due to the lack of nutrients in phagosome [26]. To overcome depletion of FeS clusters, bacteria would not only require increased levels of iron, but sulfur as well. Not surprisingly, genes involved in sulfate assimilation genes are upregulated in the *M. tuberculosis* resident within host cells, and have been discussed as antigens for future vaccine development [27]. Overall, the combined action of copper and zinc formed a 'brass dagger' in the phagosome and, without iron and manganese forming a protective shield of steel, would quickly kill bacteria. It needs to be clarified that extracellular pathogens must deal with Zn sequestration from innate immune proteins that chelate zinc [21]. On the other hand, intracellular

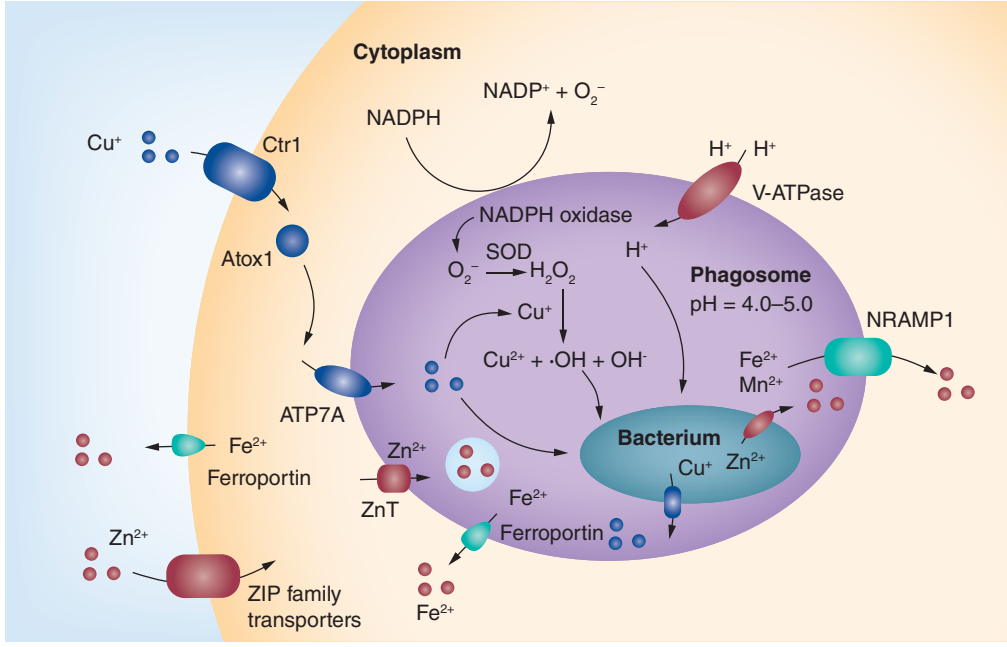

Figure 1. Metal flux and reactive oxygen production in macrophages. Membrane-bound NADPH oxidase produces hydrogen peroxide (H_2O_2), which reacts with Cu(I) and Fe(II) to generate hydroxyl radical (·OH) and hydroxyl anion (OH^-), which are highly toxic for bacteria. Cu(I) flux into cell occurs through the Ctr1 plasma membrane protein with consecutive binding to copper chaperone Atox1 in the cytoplasm, which carries Cu(I) to the P-type ATPase transporter ATP7A that is responsible for copper transport to the phagosome. ZIP family transporters allow Zn(II) penetration to cytoplasm, and CDF protein delivers zinc ions to the phagosome with subsequent storage in cellular organelles. NRAMP1 provides efflux of metals from the phagosome into cytoplasm. The resultant depletion of metal in the phagosomal environment leads to the inability of the pathogen to activate its defensive enzyme. Ferroportin plays similar role by effluxing iron ions out of phagosomal lumen, leaving the cell without the ability to maintain its energy cycle. Among many defense systems, bacteria employs multiple Cu(I), Zn(II) and Cd(II)-translocating P-type ATPases to reduce harmful concentrations in a cell.

pathogens that live and survive inside the phagosome must deal with Zn toxicity due to the amassing of intracellular Zn.

Interestingly, parts of these antimicrobial weapons are increasingly used to control hospital-acquired infections. For example, sodium hypochlorite is known to disrupt the energy cycle of bacterial cell, partially through destruction of FeS clusters, and is the active ingredient in Chlorox® (Chlorox, CA, USA). Similarly, copper surfaces covering frequently touched areas, such as door knobs, faucet handles, intravenous poles and other equipment, have shown to reduce hospital-acquired infections dramatically [28,29]. Furthermore, recent results indicate that zinc pyrithione, an active ingredient in treatment of dandruff, also promotes increased copper influx and destruction of FeS clusters in fungi [30].

What evidence suggests that copper & zinc are involved in phagosomal killing of bacteria?

In *E. coli*, a deletion of *copA* encoding a Cu(I)-translocating P-type ATPase or *zntA* encoding a Zn(II), Cd(II), Pb(II)-translocating P-type ATPase resulted in decreased survival of bacteria in macrophages [8,9]. Deletion of other genes encoding P-type ATPases, including *ctpV* encoding a putative Cu(I)-translocating P-type ATPase, and *ctpC* and *ctpG* encoding putative Zn(II)-translocating P-type ATPases in *M. tuberculosis*, gave similar results in macrophages or reduced virulence in animal models [8,31]. It should be noted that a new study defines CtcC as a Mn(II)-translocating P-type ATPase rather than Zn(II) transporter [32]. Mutations or deletions of genes encoding Cu(I)-translocating P-type ATPases and leading to reduced virulence include *ctpA* in *Listeria monocytogenes* [33], *copA* in *Streptococcus pneumonia* [34] and *cueA* in *Pseudomonas aeruginosa* [35]. In addition, a deletion of both *copA* and *golT* encoding the two Cu(I)-translocating P-type ATPases in *Salmonella enterica* sv. Typhimurium reduced survival in murine macrophages [36]. In the same organism, a deletion in *cueO* made *S. enterica* sv. Typhimurium less virulent in mice, where *cueO* encodes a multi-Cu oxidase that oxidizes Cu(I) to the less toxic Cu(II) in the periplasm [7,37].

Table 1. Putative relevant metal transporters in protozoa.

NCBI annotation	NCBI reference number	Species	Annotated transporting metal ions	Sequence similarity to	Ref.
P-type ATPase	XP_644454.1	*Dictyostelium discoideum* AX4	Copper	Copper-transporting ATPase 1 (*Homo sapiens*) NP_000043.3	[72,73]
	XP_004338204.1	*Acanthamoeba castellanii* str. Neff			[74]
	XP_004345382.1	*A. castellanii* str. Neff			[74]
	XP_638720.1	*D. discoideum* AX4			[72,73]
	XP_004339167.1	*A. castellanii* str. Neff			[74]
	XP_646096.2	*D. discoideum* AX4			[72,73]
Ctr family protein	XP_004333937.1	*A. castellanii* str. Neff	Copper	P80 protein (*D. discoideum*)	[74]
P80 protein	XP_637238.2	*D. discoideum*	Copper	Ctr family protein (*A. castellanii* str. Neff)	[75]
P80 protein putative	XP_004367963.1	*A. castellanii* str. Neff	Copper	P80 protein (*D. discoideum*)	[74]
Copper transport accessory protein	XP_004349665.1	*A. castellanii* str. Neff	Copper	P80 protein (*D. discoideum*)	[74]
NRAMP 1 homolog	XP_642974.1	*D.discoideum*	Iron and manganese	NRAMP2 (*D. discoideum*), Hypothetical protein (*A. castellanii* str. Neff)	[55,72,73]
NRAMP 2 homolog	XP_643409.1	*D. discoideum*	Iron and manganese	Hypothetical protein (*A. castellanii* str. Neff)	[72,73]
Hypothetical protein	XP_004346469.1	*A. castellanii* str. Neff	Iron and manganese	NRAMP2 (*D. discoideum*)	[74]

Ctr: Copper transporter; NCBI: National Center for Biotechnology Information; str.: Strain.

Cu-transporting channels or transporters are also required for virulence in *M. tuberculosis*. At least one more P-type ATPases, CtpG, might be involved in efflux of copper and only a double deletion of *ctpV* and *ctpG* would significantly lose virulence. Deletion of MctB (Rv1698) also had a significant effect on copper accumulation and loss of virulence, although the function of MctB is still unknown [38,39].

Copper trafficking through a P_{1B}-type ATPase and activation of the periplasmic Cu/Zn-superoxide dismutase SodCI by the periplasmic copper binding protein CueP is important for virulence [40]. Similarly, a *sodC* mutant in *M. tuberculosis* is more susceptible to periplasmic superoxide and killing by activated macrophages [41].

P. aeruginosa contains a quorum-regulated CueR-type activator that regulates a copper resistance regulon of 11 genes [42]. The main operon contains three genes encoding a resistance, nodulation, cell division (RND)-type multidrug efflux RND system, preceded by genes encoding a copper chaperone and others encoding as yet to be determined biosynthetic functions. It is possible that the copper chaperone is pumped out by the RND system to bind copper and thus prevent damage. The gene *cueA* encoding the main copper efflux P-type ATPases is also part of this regulon [42]. Many pathogenicity factors are regulated by quorum sensing [43], suggesting that the main function of many of these quorum-regulated genes is protection of cells of *P. aeruginosa* in a biofilm from protozoan grazing [44,45]. All these results clearly demonstrate a need for protection from elevated levels of Cu(I) and Zn(II) for survival within macrophages and support the view that copper resistance operons can be tied to increased virulence.

The vast majority of virulent hospital-acquired strains of *Enterobacter cloacae* possess a silver resistance determinant that is very similar to the *cus* copper resistance determinant from *E. coli* [46–48]. We suggest that rather than protecting *E. cloacae* from silver, the main function is handling copper. CusC (IbeB) in avian pathogenic *E. coli*, and by default the complete RND-type Cus system, may be involved in pathogenicity, as a CusC mutant showed significantly lower rates of meningitis in infant rats [49,50].

Do macrophages & bacteriovorus protozoa/amoeba have similar mechanisms in killing bacteria?

Several studies have shown that gene products required for survival in macrophages also work similarly in protozoa [51,52]. For the purposes of this commentary we concentrate on only a few aspects related to soft metals and oxidative burst. In *Acanthamoeba castellanii* there is production of reactive oxygen species resembling the oxidative burst of macrophages [53]. Both macrophages and at least some predatory protozoa use NRAMP-type transporters to deplete the phagosome of Fe(II) and Mn(II), as Nramp1 localize to the phagosome [54] in macrophages and in *D. discoideum* [55]. In both cells Nramp2 is also present, localizing to the contractile vacuole in *D. discoideum* or to the endosome compartment in macrophages [16,56].

The homologous Ctr1 in macrophages and P80 in *D. discoideum* are both involved in heavy membrane trafficking upon phagocytosis, and it is conceivable that both have a similar role in acquiring copper for later transport into the phagosome [7,57,58]. Both *D. discoideum* and *A. castellanii* possess at least one Ctr1 homolog characterized as P80 (Table 1).

Humans contain two genes encoding P_{1B}-type ATPases; however, in macrophages only, ATP7A regulates intracellular copper levels and pumps Cu(I) into the phagosome [59]. Silencing expression of ATP7A through iRNA led to prolonged survival of *E. coli* Δ*copA* in macrophages. *D. discoideum* harbors three genes encoding putative P_{1B}-ATPases. At least one of these P_{1B}-ATPases was responsible for the rather high copper resistance through efflux [60]. Nevertheless, antibodies against ATP7A identified that one (or more) of the *D. discoideum* P_{1B}-ATPases was not only localized in the cytoplasmic membrane, but also in vacuolar structures indicating a use not related to copper resistance [60]. Other protozoa, such as *A. castellanii*, also contain more than one P_{1B}-ATPase. The presence of multiple putative Cu(I)-translocating P-type ATPases indicates that usage is not restricted to only copper resistance and efflux. Perhaps the function of at least one of these P_{1B}-ATPases is pumping Cu(I) into the phagosome as it is in macrophages.

Are there significant differences between killing mechanisms of predatory protozoa & macrophages?

Amoeba and macrophages share many properties leading some researchers to suggest *Acanthamoeba* to be the ancestors of macrophages [61]. However, protozoa/amoeba had a much longer evolutionary history interacting with

their prey or becoming the prey themselves. So in a 'spy against spy' analogy, different strategies and counterstrategies could have resulted in different mechanisms being used in predatory protozoa to inactivate and kill bacteria. This might lead to many different solutions regarding the bacterial killing mechanism. For example, *Legionella pneumophila* contains different sets of genes necessary for survival in different protozoa [62]. *L. monocytogenes* is able to replicate in macrophages but is rapidly killed in *A. castellanii* [63]. Whether a broader reservoir of antimicrobial weapons will be a general pattern common for other predatory protozoa remains to be seen; however, it will be important to assess the potential of new species from the environment becoming potent pathogens.

Future perspective

The poisoning of intracellular FeS clusters by soft metals will be one of the key concepts in the development of novel antimicrobial strategies, leading to many possible medical applications. Already, at present antimicrobial copper surfaces are used in hospitals for effective prevention of nosocomial infections [28,64]. Such an effect is achieved through membrane damage, massive influx of copper and immediate degradation of FeS clusters [65].

Enhancing the toxicity of copper or other soft metals has the potential to become an important part of combination therapy against infections by *M. tuberculosis* and other bacterial pathogens that are difficult to treat otherwise [66]. Interestingly, silver can inhibit some proteins conferring copper resistance, such as CueO from *E. coli* [67], opening new possibilities in strategies enhancing copper toxicity. Moreover, sublethal concentrations of silver were shown to boost the effectiveness of antibiotics in Gram-negative bacteria [68]. Similar results should be obtained using gold or copper, since their mode of toxicity is expected to be similar. These results highlight the importance of understanding the whole range of antimicrobial/bactericidal mechanisms that utilize soft metals.

Historically, certain metals were used as antimicrobial drugs from the preantibiotic era [69]. We expect some of these metals or organometal compounds to be re-evaluated, perhaps in combination with antibiotics, as silver and gold nanoparticles showed promising antimicrobial activity [70,71].

Overall, metallomics of pathogenicity, innate immunity and protozoan grazing will be a rapidly growing field in the coming years with a few unexpected surprises.

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Executive summary

Soft-metal based mechanism of bacteria killing in macrophages

- Mechanisms required for regulation of intracellular concentration of soft metals were acquired by microorganisms in response to rise of the oxygen level in the environment.
- Reducing cytoplasm and presence of FeS clusters is essential for bacterial survival and present an Achilles' heel for predatory attack.
- Macrophages employ several mechanisms to kill bacteria, including oxidative burst, induction of Fe(II) and Mn(II) efflux and formation of 'brass dagger' through elevation of Cu(I) and Zn(II) levels in the phagosome.
- Defense systems in bacteria include active efflux of Cu(I), oxidation of Cu(I) to a less toxic Cu(II) form and activation of copper-binding proteins.

What evidence suggests that copper & zinc are involved in phagosomal killing of bacteria?

- Deletion of the genes encoding various Cu(I)-translocating P-type ATPases and other copper resistance determinants leads to reduced survival rate of bacteria in macrophages.
- Quorum sensing, responsible for regulation of many pathogenic factors in bacteria, is also known to influence regulation of a copper-resistance regulon in *Pseudomonas aeruginosa*.

Do macrophages & bacteriovorus protozoa/amoeba have similar mechanisms in killing bacteria?

- Mechanisms of killing in macrophages and protozoa have similar aspects, including oxidative burst, metal transporters for (Fe[II] and Mn[II]) efflux and the multiple presence of putative Cu(I)-translocating P-type ATPases.

Are there significant differences between killing mechanisms of predatory protozoa & macrophages?

- Owing to a longer evolutional history, predatory protozoa may have a bigger arsenal of bacteria-killing mechanisms.

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