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# Introduction

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The presence of a safe and adequate supply of trace metals in the environment is an evolutionary pressure that has constrained host–microbe relationships. All life forms need trace metals for nutrition and proliferation, and all organisms have evolved mechanisms to harvest the required amount of trace metals from the available pool in their particular environmental niche. This applies especially to pathogens which colonize host tissues and cause disease, and which have evolved highly efficient nutrient retrieval strategies to counteract trace metal deprivation by the host. In response to metal deprivation, pathogens elaborate elegant strategies to liberate and acquire trace metals required for growth. Additionally, under stress from trace metal deficiency, the demand of the invading parasites for the host's pool of trace metals can increase pathogenesis and disease (Abu-Kwaik and Bumann 2013). It is conceivable that these host–pathogen relationships are increasingly affected by the recent modifications of the natural biogeochemical cycles of trace metals in ways that may be significantly altering the type and levels of metals in the human host. These human-induced changes in environmental accumulation of toxic metals and depletion of essential trace elements in soils, water, and the food chain tend to be most intense in countries that have the highest burden of infectious diseases (Graham et al. 2012). We thus have an ongoing experiment of concurrent exposure of human populations to emerging metals, high levels of toxic metals and/or suboptimal amounts of essential metals, along with pathogenic organisms responsible for endemic infections in many countries. The consequences of this growing mutualism (of coexposure to metals and pathogens) on the pathogenesis of infectious diseases have been largely neglected.

Infectious diseases themselves can increase human susceptibility to the adverse effects of metal exposure, likely moderated through the immunological system and by chronic inflammation induced by the infection (Winans et al. 2011). Patients with infectious diseases can thus be considered to be at higher risk for chronic diseases because of metal exposure. Conversely, exposure to metals may aggravate diseases caused by pathogenic infections or even initiate these diseases following exposure to certain microbes. The combined effects of exposure to metals and pathogens on the burden of disease in human populations remain unknown, but they are no doubt being modified by the

increasing contamination of the environment with metals. The multiple interactions between pollutant metals, pathogens, and the environment are obviously complex and difficult to disentangle. However, in the current context of emerging metals, emerging infectious diseases, and rapid alterations of metal levels in our environment, understanding the consequences of such interactions is necessary to protect the public's health.

Many parts of the world (especially in the developing areas of Africa, Asia, and South America) that are endemic for the most common infectious diseases of our time—including malaria, HIV/AIDS, diarrhea, upper respiratory tract infections, and tuberculosis—also have high prevalence rates of trace metal deficiencies. Likewise, the highest emission rates for toxic trace metals into the environment are occurring in many countries with high incidence of infectious diseases. Coexposure to pathogens and suboptimal amounts of trace metals significantly impacts human health because trace metals command a central position at the host–pathogen interface due to the essential demand for metals that are required for many metabolic processes in all cells (Failla 2003). For microbes that reside within a niche inhabited by substantial microbial populations (such as the human gut or respiratory tract), they must compete with these other coexisting species to obtain the required amount of various trace metals. Compounding this issue is that invading microbes must also compete for these nutrients with their host. Mammals have evolved complex strategies aimed at restricting the supply of essential nutrient metals to pathogens, which represents an effective strategy of host defense sometimes termed "host tolerance" or "nutritional immunity." Note that we use the term "host tolerance" to define processes by which the host can minimize damage caused by the pathogen resulting in a homeostatic relationship between host and microbe, and this is not to be confused with "immune tolerance" which defines a state of unresponsiveness of the immune system to substances that have the potential to elicit an immune response. Notably, pathogens can evoke multiple strategies to acquire the essential element metals from their hosts to satisfy the requirement for metals in processes, including proliferation, virulence, and persistence (Failla 2003; Haase et al. 2008). The control over the homeostatic balance of essential trace metals is a critical battlefield during host–pathogen interactions which determines the course of an infectious disease in favor of either the mammalian host or the microbial invader.

A plethora of studies in recent years has revealed a complex control network of molecules involved in the competition between host cells and invading pathogens for essential trace elements. These studies have aimed at elaborating either the host's mechanisms of metal restriction or the counteracting metal acquisition strategies employed by pathogens (Inadera 2006; Jomova and Valko 2011). Recently, the concept of nutritional immunity has expanded to include host-imposed metal toxicity as a strategy to protect against microbial challenge. The most thoroughly studied of the competition for metals involves the acquisition of iron, although there is a growing appreciation of the contribution

of manganese, zinc, and copper to the outcome of host–microbe interactions. Limiting iron availability can be an efficient strategy to restrict extracellular bacteria, and such a strategy is also detrimental for intracellular pathogens. Indeed, there is now strong evidence to suggest that host-mediated alteration of iron homeostasis has direct impacts on the proliferation of microbes. Recent studies have also shown a clear-cut correlation between bacterial infections and removal of zinc from the serum. More generally, zinc deficiency can reduce immune defense against infections, chronic inflammatory disease, and reduced cellular activation, whereas high zinc can hamper effective signal transduction leading to various negative consequences. Iron homeostasis is in part linked to copper homeostasis. Copper deficiency predisposes mammals to infectious diseases, to some extent as a consequence of a lack of neutrophils induced by inadequate copper availability or supply (see Rehder et al., this volume).

Traditional wisdom holds that host-defense mechanisms are primarily a function of the immune system and can be deployed to detect and eliminate invading parasites. This paradigm has recently been challenged by studies showing that the human host uses two strategies for dealing with an infection that are not mutually exclusive: the ability (a) to limit parasite burden (resistance) and (b) to limit the harm caused by a given burden of parasites (tolerance). From an ecological perspective, resistance protects the host at the expense of the parasite, whereas tolerance saves the host from harm without having any direct negative effects on the parasite (Ayres and Schneider 2012). This distinction is useful because it recognizes the important fact that hosts can sometimes be quite healthy, despite high parasite burdens, or conversely die with parasite loads which are tolerated by others; in fact, pathogen burden and health are not always well correlated (Schneider and Ayres 2008; Ayres and Schneider 2012; Medzhitov et al. 2012). Although these two components, together, determine how well a host is protected against the effects of infection, studies of human defense against microbes have focused to date primarily on resistance; the possibility of tolerance and its implications have been comparatively less well studied (Miller et al. 2006). The linkage of metabolic cycles of trace metals to tolerance mechanisms in the host is one of the more important recent discoveries in the field of trace metals research.

Although only a few metals are known to be biologically essential (iron, molybdenum, manganese, zinc, nickel, copper, vanadium, cobalt, and selenium), there is growing realization that almost every element in the periodic table (including arsenic, bismuth, boron, cadmium, chromium, cobalt, copper, germanium, gold, iron, silver, lead, mercury, nickel, manganese, molybdenum, platinum, palladium, rhodium, ruthenium, thallium, tin, titanium, vanadium, and zinc) can also moderate a host's immune response to pathogens. Many trace metals and metalloids have been reported in human and animal experiments to display antiviral, antifungal, antibacterial, and/or antiprotozoa properties, but the mechanisms for these effects are only beginning to be uncovered.

However, the role of these metals on the tolerance defense strategies is currently unknown.

A close look at the modes of death from infections shows surprisingly that death is often not attributable to a direct effect of the pathogen or of any toxin it produces but rather is the consequence of the systemic inflammatory response in the host (Baillie 2014). Our own immune system can be responsible for destroying us. Although this has been known for some time, efforts to find effective therapies that alter the host response to infection to promote survival have not been tremendously successful. This failure may be related, in part, to the inability to distinguish between failed resistance and failed tolerance in monitoring the outcome of treatment. When failed tolerance is the underlying factor, boosting immunity and reducing pathogen burden (using drugs) may be ineffective, whereas enhancing tolerance (e.g., with trace metal intervention) may have salutary effects. Drug interventions that target tolerance pathways may also be more desirable when immune defenses are either inefficient, compromised, or cause excessive immunopathology. Boosting tissue tolerance could be a particularly useful strategy in diseases such as malaria, tuberculosis, and HIV, where pathogen control through vaccination or antimicrobial drugs is currently suboptimal (Medzhitov et al. 2012). Conceptually, it is appealing to reduce the effect of an infection by moderating the host environment as opposed to poisoning the pathogen with a toxic metal. In this sense, metallic compounds hold some promise as potentially effective, adequate, affordable, and safe chemotherapies to boost host tolerance as well as resistance.

Unlike the essential metals, exposure to many toxic heavy metals found in the environment may trigger autoimmunity (overactive immune system) or result in immunotoxicity (Dietert 2009). The manifestation of autoimmune diseases includes production of autoantibodies, inflammation and cytokines in various target organs, and deposition of immune complexes in vascular sites (i.e., immunopathology). At high enough doses, exposure to metals can exert direct toxicity on the immune system through suppression of the system as a whole or by the disruption (suppression) of immunoregulatory systems, and hence result in exaggerated responses to infections (Failla 2003). In terms of the combined effect of coexposure to essential and toxic metals, it is conceivable for the antibiological activity of one metal to amplify the activity of a distinct metal, leading to increased resistance (or tolerance) against infections. A few *in vivo* and *in vitro* studies have reported reduced host vulnerability in response to coexposure to two or more trace metals. On the other hand, the antibiological activity for one metal can be incompatible with the activity of another metal, which then can lead to enhanced virulence or morbidity following an infection. Competition phenomena between zinc and several trace metals (such as cadmium, lead, calcium, iron, manganese, and copper) have been documented in zinc supplementation trials; however, results are inconsistent because of failure to consider the basal zinc status during the experiments. There are some parts of the world where communities with high prevalence

of iron, zinc, or selenium deficiency are being exposed to high levels of toxic metals (especially lead, mercury, and arsenic). Populations in such areas are well suited for epidemiological studies aimed at understanding the underlying mechanisms of how trace metal interactions moderate the outcome of an infection.

## General Perspectives from the Forum's Discussions

Interest in processes at the nexus of host–microbe–metal interactions has risen recently as a result of advancements in the study of metallomics (metal-containing biomolecules), proteomics, and genomics. These emerging fields have given rise to new developments in powerful analytical methods and technology for studying the identity, distribution, quantity, trafficking, fate, and effects of trace metals in biological systems. Applications of these advanced techniques to the study of metabolic cycles are yielding results and have placed scientists at the threshold of major paradigm shifts in our understanding of the relationships between homeostatic mechanisms of trace metals and pathogenesis of infectious diseases. This emerging field was thus well suited for an Ernst Strüngmann Forum, which applies a unique multidisciplinary framework to assess current knowledge and identify gaps and research opportunities on cross-cutting issues. The fields present at this Forum were broad: chemistry, biology/biochemistry, toxicology, nutrition, immunology, microbiology, epidemiology, environmental and occupational health, as well as environmental and veterinary medicine. The majority of participants shared common interests in the roles of metals in biology, and they were tasked with using their knowledge to discuss and create reports on how the metabolic cycles of trace metals relate to the pathogenesis of disease during infection. To prepare for this discussion, invited background papers provided reviews of critical topics as a basis for the group discussions. The stimulating dialog that ensued covered a wide range of views, insights, and perspectives on current knowledge and raised important open questions that should be addressed by future research initiatives. Detailed summaries of the current state of knowledge and future areas for further research and development are presented in the group reports. The overviews below are our perceptions of some of the general areas of concern expressed at the Forum.

Cavet et al. (Chapter 7, this volume) focus on the microbial perspective during host–microbe interactions. Their discussions were directed at five key areas deemed to require greater understanding:

1. Metal availability in distinct environments, including within and outside microbial cells, with some emphasis on intracellular metal availability and how metal-requiring proteins acquire their correct metal cofactors.

2. The different levels and sources of metals available to microbes in distinct niches within the host.
3. The effect of the metal status of a pathogen, as derived from its prior environment, on its ability to establish an infection or the severity of disease.
4. The interplay between metals and the microbiota.
5. How metal restriction and metal oversupply can inhibit microbial growth or cause their death.

Specific issues that are explored in detail by Cavet et al. include the nature of the pool of exchangeable metal inside cells; whether metalloforms of an enzyme differ depending upon circumstance; control of metallation status by thermodynamics of binding sites; metabolic metal shuttling (metal ligands and proteins); the diversity of ecological niches for microbes in the vertebrate host; the sources and forms of metals in different niches; metal speciation in the intracellular environment; microbial strategies for obtaining metals from host and the effects of metal restriction; how an overabundance of intracellular metals impedes cell growth; and the effect of metal import and export systems on microbial growth and pathogenesis. They also cover some of the most intense and exciting areas of research on metals in the biology of microbes.

Rehder et al. (Chapter 13, this volume) provide a comprehensive overview of the role of metal ions in infectious diseases from the host perspective, focusing on iron, copper, zinc and, to a lesser extent, manganese and the metalloid selenium. Additionally, recommended dietary allowances (RDAs) are addressed, as well as metal-based drugs in the treatment of tropical diseases. The issues highlighted by Rehder et al. include the roles of manganese, iron, copper, zinc, and selenium in immune function; the interplay of iron distribution between microbes and host cells; the impact of iron on anti-immune effector functions; the role of zinc in host resistance/susceptibility and tolerance; therapeutic effects of zinc in infectious diseases of children; the specific case of selenium and susceptibility to viral and bacterial infections and to parasites; and a commentary on the RDAs and related intake levels. Their report includes a discussion of the role of the gasotransmitters carbon monoxide and nitric oxide in relation to their interference with bound and free metal ions. To cope with the increasing concern about the epidemic of tropical diseases (such as leishmaniasis, Chagas disease, and malaria), drugs are being developed that are based on coordination compounds of metals, including copper, iron, ruthenium, and gold. The efficacies and limitations of such drugs are described by Rehder et al. This report synthesizes the current state of knowledge regarding the contribution of metals to infectious disease from the host perspective and offers recommendations for areas of future research.

Ackland et al. (Chapter 17, this volume) provide insights into current knowledge and gaps in our understanding of the interplay between trace metals in the environment and infection. The contributions of metal deficiencies

to the global burden of infectious diseases are substantial for zinc and iron but less defined for other metals. How emerging metals relate to emerging pathogens, and hence influence the disease burden, is identified as a matter that deserves further research. Despite considerable research taking place separately on trace metals and infectious pathogens, little is currently known about the interactions between these two key determinants of health, especially in the host microbiota, where direct coexposure occurs. A number of global trends have been identified that have the potential to upset the natural host–microbe–metal nexus, including climate change, Western-style food processing, increasing reliance on infant formula and consumption of fast foods, as well as the commercialization of products with metalliferous nanomaterials. From an ecological perspective, the two main processes by which the environment can directly impact host–pathogen interactions are (a) the changing pathogenicity of infectious agents combined with the emergence and spread of drug resistance, and (b) the changing of host resistance to the pathogen. The contributions of metals to the specific mechanisms involved in each process are essentially unknown.

Ackland et al. note that failure to ascertain the environmental contribution to infectious disease etiology stems largely from limitations in our ability to assess the environmental exposures, which have traditionally been measured using questionnaires and geographical mapping. The need for a new exposure paradigm that can integrate many external and internal exposures from different sources over the life course is emphasized. Exposomics or environment-wide association studies offers one such approach to gain insight into the environmental component essential to improving our understanding of the predictors, risk factors, and protective factors in complex interactions between trace metals, the environment, and infective microbes. An understanding of the effects of such an environmentally determined exposome on susceptibility to infectious disease would be an important step in developing appropriate intervention strategies in many parts of the world.

Our understanding of the biology of metals in the context of infectious disease is necessarily advanced (and limited) by the available analytical tools. The broad aims of Maret et al. (Chapter 20, this volume) were to provide an overview of analytical techniques available for investigating the interaction of metals/metalloids within both microbe and host, to specify needs for technological improvements, and to identify emerging applications and analytical questions. Measurement of metals in biological samples should encompass the total metal content, chemical speciation of metals, and additional information about distribution in biological space and time. Maret et al. stress the need to generate data that can be used to understand the metallome, or the functions of all parts of systems biology. In their report, they identify gaps and needs in technology, upcoming methodological issues, and analytical ways to study therapeutic and preventive interventions that will address the host–microbe interaction, with a focus on further goals and potential applications.

In this regard, Maret et al. discuss the adaptation of hyphenated techniques in proteomics research that require protein separation, mostly chromatographic and electrophoretic, in combination with molecular mass spectrometry (ICP-MS, ES-MS, MALDI-MS). In addition, the pool of nonprotein-bound metals, which is increasingly drawing attention because it is "metabolically active," can be addressed with fluorimetric techniques that have detection limits down to the septomolar range of concentrations or even reach single molecule or single cell resolution with super-resolution microscopy. An important issue for further research is the development of isotope tracer techniques, which can be used in experimental designs that span a wide range due to the large differences in size, reproductive speed, and experimental tractability of microbes and host animals. Microbes and cultured cells can be grown in media containing radioactive or stable isotope tracers, and the total inventory of metal species can theoretically be identified and quantified by radioactivity detection or by the isotopic shift, respectively. In higher animals, tracer techniques allow application of uptake measurements and metal species turnover, but lack or bias the information on the total inventory of metals. Currently, localization and speciation of total metals or the metallome in higher animals is pursued only *ex vivo* in tissue sections or extracts of cells, tissues, or body fluids because many of these techniques are destructive in nature.

Significant insight into trace metals and infections can be gained from further development and improvement in the transgenic constructs of metal sensors/reporter proteins from the cellular to the organismal level. Permanent or conditional knockouts (KO) of metal chaperones, transporters, chelators, or storage proteins elucidate the mechanism of metal-related host defense, but typically require the use of KO mice. Fusions of fluorescent or luminescent proteins with metal-chelating proteins may report free metal ion concentrations. Luminescent reporter proteins expressed under the control of promoters for genes of interest allow for studying the regulation of metalloproteins or effector proteins responsive to metals and can provide an indication of the metal environment experienced by a pathogen during infection. In addition, Maret et al. review *in silico* approaches to metalloprotein function to predict and calculate the dynamics of metallomes for organisms, starting from their genome sequences. Attention is drawn to existing or upcoming genome-wide expression studies utilizing real-time polymerase chain reaction for identifying genes encoding metalloproteins, which are up- or down-regulated in the host or microbe upon mutual contact or may mediate metal modulation of infectious disease.

In their report, Maret et al. include a general overview of existing analytical methods for trace metals; in particular, inductively coupled plasma mass spectrometry (ICP-MS), which has been established as the workhorse in non-radioactive metal speciation studies and, when coupled to a laser ablation (LA) instrument, enables imaging at the mesoscopic scale. ICP-MS provides the lowest detection limits and the best sensitivities, highest concentration

dynamic range, broad multiplex capability, and robustness toward matrix effects. Identification of metalloproteins after chromatographic or electrophoretic separation uses metal detection by ICP-MS (or autoradiography) and for protein determination by tryptic digestion, followed by high-resolution tandem mass spectroscopy. The experimentally determined amino acid sequences of the peptides are then compared with those organized in protein databases, yielding acceptable or unacceptable probabilities of correct identifications. Unfortunately, protein databases lack many metalloproteins and contain numerous mis-assignments of metalloproteins, which is a major shortcoming in the field of metallomics.

In summary, through this Forum we collectively sought to expand understanding of the linkage(s) between metals and the pathogenesis of infectious diseases, and to address the underlying mechanisms that moderate the outcome of infection. The dialog that emerged from a tremendously diverse group of international experts attests to the very real needs that exist in science. This volume aims to transfer the Forum's results. It provides an integrated summary of our current knowledge, highlights the contentious issues, and suggests critical areas for further research. We hope that it will spur future work and discovery in the service of humankind to alleviate the burden of infectious disease throughout the world.

The outcome of the Forum depended very much on the efficiency, helpfulness, and general guidance of Julia Lupp and the entire staff of the Strüngmann Forum. Their unflinching support and professionalism were appreciated by all who attended the meeting. The Forum benefited greatly from the work of the Program Advisory Committee members—Rodney R. Dietert, Julia Lupp, Jerome O. Nriagu, Lothar Rink, Anthony B. Schryvers, and Eric P. Skaar—who met in Frankfurt from January 26–28, 2013 to develop the scientific program for the Forum. Success, however, belongs to the moderators, rapporteurs, and all participants. In closing, we wish to thank the authors of the invited background papers as well as the rapporteurs, who share credit for the quality of material in this book.