IMPACT OF MACROPHAGE ZINC METABOLISM ON HOST DEFENSE AGAINST MYCOBACTERIUM TUBERCULOSIS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

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ABSTRACT

Tuberculosis (TB) is a global epidemic caused by infection of human macrophages with the world’s most deadly single bacterial pathogen *Mycobacterium tuberculosis* (*M.*tb). Manipulation of dietary micronutrients is a critical mechanism of host defense against infection and is referred to as nutritional immunity. In particular the essential trace element zinc functions as a critical modulator in inflammation through the human zinc transporter ZIP8. We hypothesize that zinc metabolism modulates macrophage host defense through ZIP8 during infection with *Mycobacterium tuberculosis* which is critical to the host response to TB.

We began our investigation by establishing a physiologically relevant, *in vitro* model for the evaluation of zinc metabolism in human macrophage host defense. We then used the model to investigate the relationship between zinc, ZIP8 and NF-κB. ZIP8 is constitutively present in human macrophages, and induced through NF-κB as well as in response to LPS. Cellular zinc deprivation of macrophages increases LPS-induced macrophage zinc uptake, however ZIP8 knockdown in macrophages does not impact zinc accumulation prior to the arrival of the induced protein. Zinc supplementation increases NF-κB activity,
independently of ZIP8. However, in response to LPS, ZIP8 inhibits NF-κB in the absence of zinc supplementation.

Based upon these observations, we next identified the impact of ZIP8-dependent zinc on the balance of macrophage pro- and anti-inflammatory cytokine production. Zinc uptake within hours of LPS stimulation results in reduced IL-10 production and an increase in pro-inflammatory cytokine production in macrophages. ZIP8 knockdown partially reverses zinc-dependent reduction of IL-10 release but does not impact pro-inflammatory cytokine production. Localization of NF-κB subunits to binding sites on the IL-10 promoter is independent of zinc and ZIP8. However zinc supplementation of LPS-exposed macrophages reduces activity of the IL-10 inducing transcription factor C/EBPβ revealing a potential mechanism for the IL-10 effect.

Finally we identified the impact of zinc and ZIP8 on macrophage inflammation and host defense during *M.tb* infection. We began by critically evaluating published epidemiologic studies and discovered that TB disease correlates with inadequate nutritional zinc intake and reduced circulating zinc levels. We also determined *in vitro* that *M.tb* infection uniquely induces ZIP8 expression in comparison to the other 23 known zinc transporters. *M.tb* infection during zinc supplementation results in redistribution and increase of cytosolic zinc. Importantly, supplementation of zinc during macrophage infection reduces both IL-10 production and *M.tb* growth, which is independent of ZIP8. Using a novel, *in vivo* myeloid specific ZIP8 knockout mouse we determined that during
*M.tb* infection, ZIP8 induces expression of pro-inflammatory cytokines and immune modulators in alveolar macrophages. Finally we revealed that the absence of ZIP8 is associated with decreased *M.tb* growth in the lung *in vivo*. This work establishes a framework demonstrating that macrophage zinc metabolism modulates the antimicrobial response to *M.tb* through alterations in zinc metabolism. The impact of zinc metabolism on *M.tb* infection is both ZIP8-dependent- and –independent, further revealing that the impact of zinc is complex, involving multiple factors. These critical observations will facilitate investigation of the impact of macrophage zinc metabolism on the intracellular lifestyle of *M.tb*. Future studies determining the relative cellular distributions of ZIP8 and zinc throughout infection and the mechanisms underlying their impact on critical host defense functions have the potential to improve our understanding of TB pathogenesis.
DEDICATION

This document is dedicated to my daughters Scarlet and Alannah.
ACKNOWLEDGMENTS

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PUBLICATIONS


FIELDS OF STUDY

Major Field: Pharmaceutical Sciences
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<tr>
<td>AE</td>
<td>Acrodermatitis enteropathica</td>
</tr>
<tr>
<td>AAS</td>
<td>Atomic absorption spectroscopy</td>
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<tr>
<td>AES</td>
<td>Atomic emission spectroscopy</td>
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<td>AKT</td>
<td>Protein kinase B</td>
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<td>AM</td>
<td>Alveolar macrophages</td>
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<tr>
<td>ATP7A</td>
<td>Menkes’ protein P-Type ATPase copper transporter</td>
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<tr>
<td>BCG</td>
<td><em>Mycobacterium bovis</em> Bacillus Calmette–Guérin</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin histone immuneprecipitation</td>
</tr>
<tr>
<td>C/EBPβ</td>
<td>CCAAT/enhancer-binding protein beta</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>CLP</td>
<td>Cecal ligation and puncture</td>
</tr>
<tr>
<td>CTR1</td>
<td>High affinity copper uptake protein 1</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>Cu</td>
<td>Copper</td>
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<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DRI</td>
<td>Dietary reference intake</td>
</tr>
<tr>
<td>DTPA</td>
<td>Diethylenetriamine pentaacetate</td>
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<tr>
<td>E.coli</td>
<td>Escherichia coli</td>
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<tr>
<td>EE</td>
<td>Environmental enteropathy</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases 1 and 2</td>
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<tr>
<td>g</td>
<td>Grams</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony-stimulating factor</td>
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<td>GMDP</td>
<td>N-glycolyl-muramyl dipeptide</td>
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<td>GSH</td>
<td>Glutathione</td>
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<td>H.capsulatum</td>
<td>Histoplasma capsulatum</td>
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<td>IFN-γ</td>
<td>Interferon gamma</td>
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<td>IKK</td>
<td>IκB kinase</td>
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<td>IL-1β</td>
<td>Interleukin 1 beta</td>
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<td>Interleukin 2</td>
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<td>Interleukin 8</td>
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<tr>
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<td>Interleukin 10</td>
</tr>
<tr>
<td>LAM</td>
<td>Lipoarabinomannan</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<td>Abbreviation</td>
<td>Term</td>
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<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td><em>M. tb</em></td>
<td><em>Mycobacterium tuberculosis</em></td>
</tr>
<tr>
<td>ManLAM</td>
<td>Lipoarabinomannan</td>
</tr>
<tr>
<td>MDM</td>
<td>Monocyte-derived macrophage</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug-resistant</td>
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<tr>
<td>MEK</td>
<td>MAPK/ERK kinase</td>
</tr>
<tr>
<td>MHCII</td>
<td>Major histocompatibility complex II</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Manganese superoxide dismutase</td>
</tr>
<tr>
<td>MDP</td>
<td>Muramyl dipeptide</td>
</tr>
<tr>
<td>MRE</td>
<td>Metal response element</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MTF-1</td>
<td>Metal-response element binding transcription factor-1</td>
</tr>
<tr>
<td>MctB</td>
<td>Mycobacterial copper transport protein B</td>
</tr>
<tr>
<td>NRAMP1</td>
<td>Natural resistance-associated macrophage protein 1</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOD2</td>
<td>Nucleotide-binding oligomerization domain-containing protein 2</td>
</tr>
<tr>
<td>Nrf-2</td>
<td>Nuclear factor, erythroid 2 like 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>p38</td>
<td>p38 mitogen activated protein kinase</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
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<tr>
<td>pM</td>
<td>Picomolar</td>
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<tr>
<td>PEM</td>
<td>Protein energy malnutrition</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PTPRC</td>
<td>Receptor-type tyrosine-protein phosphatase C</td>
</tr>
<tr>
<td>RDA</td>
<td>Recommended daily allowance</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>S.Typhimurium</td>
<td><em>Salmonella typhimurium</em></td>
</tr>
<tr>
<td>SLC11A</td>
<td>Solute carrier family 11A</td>
</tr>
<tr>
<td>SLC30A</td>
<td>Solute carrier family 30A</td>
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<td>SLC39A</td>
<td>Solute carrier family 39A</td>
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<td>S.aureus</td>
<td><em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>SOCE</td>
<td>Store operated calcium release</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TPEN</td>
<td>N,N',N',N-tetrakis-(2-pyridylmethyl)-ethylenediamine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>TGN</td>
<td>TransGolgi network</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>Th₁</td>
<td>Type I T helper cells</td>
</tr>
<tr>
<td>THP1</td>
<td>Human monocytic leukemia cell line</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>UL</td>
<td>Tolerable Upper Intake Level</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adapter-inducing interferon beta</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>XDR</td>
<td>Extensively drug-resistant</td>
</tr>
<tr>
<td>Vₘ</td>
<td>Membrane potential</td>
</tr>
<tr>
<td>ZIP</td>
<td>Zrt-Irt-like-Protein zinc transporters</td>
</tr>
<tr>
<td>ZnT</td>
<td>Zinc Transporter</td>
</tr>
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CHAPTER 1: INTRODUCTION

1.1. General Introduction

The goal of this dissertation is to determine the impact of the micronutrient trace element zinc and its metabolism within human macrophages on host defense during Mycobacterium tuberculosis (M.tb) infection. The studies herein focus primarily upon the regulation of zinc trafficking through the zinc import protein ZIP8 (SLC39A8) and its impact on macrophage defense functions. This work utilizes innovative scientific approaches to comprehensively evaluate how zinc influences tuberculosis (TB) pathogenesis at the host-microbe interface. It bridges the disciplines of nutrition, innate immunity, inflammation, microbiology and infectious disease pathogenesis to uniquely address these critical questions. This research builds important knowledge relevant to human health and disease and reveals insights into potential novel zinc-based therapeutic strategies. The following introductory chapter will provide relevant conceptual background information on nutrition, its function in immunity as well as details encompassing zinc metabolism, TB pathogenesis and how the two impact one another.
1.2. Nutrients in Health

The Nutritional Imperative

Appropriate intake of nourishment is required for normal health and indispensable to all forms of life irrespective of taxonomy, ranging from primitive prokaryotic microorganisms to modern humans. Satisfaction of nutritional intake requirements is among the principal forces that govern normal cellular behavior and ultimately the existence of every organism. The pressures of scarcity and competition for nutrition are defining features of the ecological niche and provide context for interactions between and among species [1]. These interactions are exemplified within the microcosm of host-microbe interactions. Colonization of the host as a microbial niche is predicated in part upon the ability of microorganisms to derive nutrition from the host at a cellular level within a survivable range. In fact the basic function of both microbial pathogenicity and host defense is often manipulation of nutrient resources [2]. Functionally, replenishment of nutrients conserves homeostasis, promotes growth and development and fosters survival by increasing adaptability to internal and environmental pressures at every level of cellular organization.

Complex metabolic networks facilitate nutrient utilization through sequential chemical transformations evolved to exploit the intrinsic physical and chemical properties of these distinct elements in support of life-sustaining functions. Paradoxically, adequate nutrient consumption supports life largely by satisfying the demands of metabolism, which have evolved as a response to
historical environmental nutrient availability. The anabolic and catabolic processes that constitute metabolism utilize individual nutrients to generate precursors of cellular growth and maintenance, liberate caloric energy from organic compounds, eliminate byproducts and support specialized cellular activities. Specific nutrients mediate cellular activity by acting as cofactors and substrates for numerous critical functions including gene transcription, RNA translation as well as post secondary protein modification, stability and degradation. Accordingly metabolic trafficking of nutrients is an essential signaling mechanism by which environmental inputs regulate physiological responses. Thus, in addition to nutrient availability, appropriate nutritional balance is necessary to sustain metabolic equilibrium in order to maintain cellular function and physiological homeostasis.

Dietary requirements are determined by the bioavailability and biodistribution of a nutrient, its functional demand and metabolism as well as its rate and route of elimination. It follows that requirements are altered by factors like gender, age, growth and development. The recommended dietary allowance (RDA) of a nutrient is formulated to be adequate to meet the needs of practically all (97.5%) healthy persons of similar age and gender without consideration to those with special nutritional needs. However, although adequate, these recommendations may not meet requirements for optimal physiological effect, which are more accurately represented by dietary reference intake (DRI) recommendations [3]. Nutrient deficiency can result from inadequate intake or
disease and infection, which may also lead to malabsorption. Additionally, nutrient malabsorption may be caused by genetic disorders or co-consumption of nutrient binding factors, medications or dietary constituents such as phytic and oxalic acids in the case of divalent cations. Modification of dietary nutrient intake or supplementation is often necessary in deprived or pathophysiological states. However, determination of appropriate doses for supplementation during malnutrition or disease is often problematic for nutrients present at low levels. This is the case for most micronutrients, including zinc, for which blood levels are not reflective of nutrient concentration in target tissues. Further, toxicity is often associated with acute or chronic nutrient over consumption, which is of particular concern with over supplementation. The maximal level of chronic consumption that is unlikely to adversely effect health defines the tolerable upper intake level (UL) of daily nutrient intake, which is a general guideline for limits on nutrient supplementation [3].

Dietary supplements or food fortification are often required to counteract the global epidemic of chronic nutritional deficiency associated with malnutrition caused by economic disparity. Nutrient deficiencies are classified according to the physiological response associated with their dietary inadequacy. Type I nutrient deficiency is characterized by specific physical signs of deficiency that result from reduced tissue concentrations of the nutrient following reductions in bodily stores, such as blindness due to Vitamin A deficiency. A type II nutrient deficiency lacks specific deficiency signs but is characterized by a generally
reduced growth rate and weight loss such as that associated with inadequate caloric intake [4]. Malnutrition generally compromises essential bodily functions including immunological host defense. Consequently, pathogenic infection is likely to be more severe in the setting of malnutrition and also prone to perpetuate nutritional deficits that are cumulatively more deleterious to the host [5]. Human nutritional requirements encompass a wide array of compounds and elements each with a unique contribution to human health. The major constituents of human nutrition are categorized according to their relative dietary demand and physiological requirements [4].

Macronutrients, Water & Macrominerals

Macronutrients are required in gram quantities in the human diet and include carbohydrates, lipids and proteins. They are organic compounds utilized principally as calorie sources toward the formation of high-energy phosphate bonds following consumption, digestion, absorption and distribution. Macronutrient malnutrition results in type II deficiency. Carbohydrates, which are composed of monomers or polymers of simple sugars, are the major short-term energy source for cellular respiration. Lipids are a diverse group of hydrophobic compounds composed primarily of non-polar hydrocarbons that are a secondary source for cellular energy but also serve as the core energy storage reserve and provide constituents for cell structure and function. Proteins are made up of sequences of amino acids, which provide nitrogen in a bioactive form. Protein
consumption is required for the formation of nitrogen containing molecules including human proteins that are assembled from dietary and newly synthesized amino acids. Additionally, proteins are utilized as an energy source when carbohydrates and lipids are in short supply [4, 6].

Water is the most abundant substance within the human body. It is ubiquitously present as an electrolyte solution. Water permeates cell membranes through aquaporin channels and constitutes the medium in which nutrients are solubilized and in which extracellular and intracellular metabolic reactions occur. The polar nature of water dictates the context of nutrient interactions within the body based upon their relative charge or hydrophobicity. Proper electrolyte balance within body water is essential to homeostatic water distribution, pH regulation and osmolarity. The compartmentalization of water depends upon electrolyte concentrations within cells and cell compartments as well as in the interstitium or vasculature. Proper water consumption and balance are essential to homeostasis and cellular function. Severe dehydration even in the short term is incompatible with life, which manifests as a type II deficiency [4].

Macrominerals are elements required in the diet in amounts greater than 100 mg daily that facilitate signal transduction, promote chemical reactions, shape chemical structures and maintain bodily function. They are charged water-soluble electrolytes that include cations: calcium (Ca$^{2+}$), magnesium (Mg$^{2+}$), sodium (Na$^+$), potassium (K$^+$) as well as anions: phosphorus (P$^{3-}$), sulfur (S$^{2-}$), and chloride (Cl$^-$). Calcium and phosphorus are present in the largest quantities
in the body due to their crystallized deposition for structural support in bone. Ionic charge and relative abundance endows macrominerals with the ability to establish and depolarize electrochemical gradients across non-polar plasma membranes, which is the foundation for many cellular transporters and all neurological signaling and muscular contraction [6]. The valence of nutritional minerals often determines their utility and function. Divalent cations including calcium and magnesium serve both in cellular polarization and signaling as second messengers whereas monovalent cations such as sodium and potassium are largely limited to regulation of membrane potential changes [7]. Intracellular signaling by cations is dependent upon extreme restriction of unbound labile cytosolic concentrations through efflux pumps, protein binding or sequestration within intracellular membrane bound compartments. For example, in leukocytes calcium signaling is initiated when receptor activation triggers calcium release from intracellular stores generating transient intracellular calcium waves. Those calcium waves are critical to T cell, macrophage and dendritic cell host defense responses [8]. Calcium malnutrition results in type I deficiency characterized by inadequate mineralization of bone and muscle tetany.

**Micronutrients**

Micronutrients are essential components of human nutrition required in the diet in amounts less than 100 mg daily. They are categorized as either vitamins or trace elements. Vitamins are organic compounds for which physiological
synthesis is absent or insufficient to meet metabolic demands. Interestingly, Vitamin D is also categorized as a pro-hormone as its precursors can be synthesized from cholesterol then activated by UVB sunlight but must be ingested in the diet during low light conditions. Trace elements are micronutrient mineral elements with bioactivities that rely primarily on their inherent ionic charge. Micronutrient deficiencies are all type I with specific signs and symptoms except for zinc deficiency, which results in type II growth defects.

Vitamins are classified as fat-soluble or water-soluble according to their ability to dissolve in non-polar or polar solvents. The fat-soluble vitamins include vitamins A, D, E and K. Due to their non-polar nature, intestinal absorption and vascular circulation of fat-soluble vitamins requires co-distribution with lipids in micelles and blood lipoproteins respectively. They are stored in the body due to their lipid solubility. Metabolites of vitamin A (retinoic acids) and vitamin D (calcitriol) are important regulators of gene expression as ligands for the RAR and RXR or the VDR transcription factors respectively. Vitamin A is essential to light absorption in vision, developmental organogenesis, tissue repair and lymphocyte differentiation. Vitamin D is critical for calcium and phosphorus balance, differentiation, cell cycle arrest as well as for adaptive and innate immune function. Vitamin E acts primarily as a free radical scavenging antioxidant whereas vitamin K is a critical cofactor in the synthesis of components of the coagulation cascade. The water-soluble vitamins include the B vitamins: B1 (Thiamin), B2 (Riboflavin), B3 (Niacin), Pantothenic acid, B6
(pyridoxine, pyridoxal, pyridoxamine), Biotin, Folic acid and B12 (cobalmin) as well as vitamin C. Due to their solubility they cannot be retained in the body for long periods and are lost to renal excretion with the exception of B12 which is eliminated in the bile. The B vitamins function as coenzymes mainly in processes that support energy metabolism. The primary physiological function of vitamin C is as an antioxidant reducing agent and enzymatic cofactor.

Trace Element (micromineral) classification depends upon their daily requirement and relative abundance in the body. Copper, iron, manganese and zinc are essential trace elements whereas selenium, iodine, arsenic, chromium, cobalt, fluorine, molybdenum, nickel, silicon and vanadium are considered essential ultra-trace elements due to their comparatively smaller daily dietary demand, which is in the microgram range [9]. In general they play indispensable roles in protein structure and as enzymatic prosthetic groups or cofactors. Similar to macrominerals the absorption, biodistribution, bioactivity and elimination of trace elements is predicated largely upon their ionic charge. The presence of one divalent cation can impact bioactivity of others by modulating their absorption and metabolism. Supplementation of manganese reduces iron absorption [10] whereas iron can interfere with zinc absorption and visa versa [11] and zinc supplementation can reduce copper absorption [12]. Furthermore, iron or copper but not zinc can each exist in two different oxidation states in physiological conditions, which facilitates many of their biological functions in redox reaction catalysis [4]. Iron is a critical cofactor for oxygen transport and storage and
promotes oxidative phosphorylation during anti-microbial innate immune defense [13, 14]. Copper and copper containing cuproenzymes are required for a diverse array of physiological functions including but not limited to iron metabolism, energy metabolism, reactive oxygen species detoxification and anti-microbial host defense [15, 16]. Manganese is critical for cartilage formation and as a cofactor for metalloenzymes. Zinc is the focus of this research and is reviewed in detail below.
1.3. Zinc

Physiological properties of zinc

The physiological essentiality and biophysical properties of zinc make it unique among micronutrients and trace elements. Zinc is an indispensable structural, catalytic and regulatory protein cofactor. The unique complexation chemistry of zinc with the amino acids histidine, glutamate, aspartate, and cysteine gives it both strong and exchangeable ligand binding properties. As a result it has the ability to irreversibly bind protein structural components or catalytic cofactors but also regulates signaling, trafficking and storage through reversible protein bonds [17]. Zinc is required in up to 10% of the human proteome. Zinc fingers are necessary structural components of over 2500 proteins and more than 300 metalloenzymes that require zinc as a catalyst for activity [18]. Zinc is only physiologically present in its ionic Zn$^{2+}$ oxidation state. Accordingly, it is not directly involved in physiological redox reactions but can act as an ancillary antioxidant factor. Accordingly, zinc supplementation reduces markers of oxidant stress in human volunteers [19]. It shields protein thiol groups from reactive oxygen species and reduces intracellular reactive oxygen species formation. Zinc prevents the interaction of iron and copper with proteins thereby limiting damage from localized Fenton and Haber-Weiss reactions [20]. Zinc has a higher protein binding affinity than most other physiologically relevant divalent cations including calcium and magnesium as well as the other transition metals excluding copper. This allows it to displace other physiological metals and
typically prevents its own displacement [21]. Additionally, due to its charge, zinc is unable to cross phospholipid bilayers by passive diffusion. Therefore trafficking into, out of, and between membrane bound vesicles of cells requires protein transporters. As a result zinc metabolism is exquisitely orchestrated. Zinc regulates gene expression through direct activation of transcription factors and also indirectly by regulating intracellular phosphorylation mechanisms, which modulate transcription factor activation. This ability to quickly alter cellular response as a second messenger affords zinc a particularly critical role in host defense.

The human body contains 2-3 g of zinc at any given time, which is primarily intracellular (95%), and requires continual dietary replenishment to meet metabolic demand [22]. Dietary zinc is absorbed by enterocytes throughout the intestinal tract with its highest rate of absorption in the jejunum [23]. It is then trafficked into circulation via the portal vein, transiently elevating serum concentrations before being absorbed in remote tissues. The majority of human zinc is contained within bone and muscle [24]. However, zinc is present in all body fluids as well as tissues, where approximately 10% is exchangeable with the plasma [25]. Serum zinc concentrations vary between 10-18 µM in the human body [26] and approximately 70% is bound to albumin. The remainder of circulating zinc is tightly bound to other proteins including α-2 microglobulin and transferrin [27]. Plasma proteins buffer ionic zinc in the vasculature but are not a tractable reservoir for storage. There is no specific storage source for zinc but
cells maintain a small transient pool of zinc within intracellular vesicles that may exchange with plasma in times of need [28]. A sufficient state is achieved when the RDA for zinc is met. Adult men require 11 mg and adult women require 8 mg daily [29] with an associated UL of 40 mg daily. Unfortunately accurate determination of zinc status from the plasma is difficult due to inter-individual variability and mobilization of zinc out of the plasma compartment during acute stress and infection [30]. Combinatorial evaluation of zinc intake, plasma level and developmental indices in children are thought to be the most definitive diagnostic tools for human zinc status [31]. Potential alternative methods for measuring zinc status are zinc-dependent enzymatic activity, cellular responses and tissue content although none of these approaches are conventionally used in the clinical setting.

Determination of intracellular zinc status presents its own set of unique challenges, particularly assaying levels of rapidly exchangeable functional pools. Cells contain zinc between 200-300 µM with practically all zinc protein bound or contained within vesicles. Steady state labile intracellular free zinc concentrations are in the picomolar (pM) range and transient increases are rapidly sequestered into subcellular reserves, typically in the nanomolar (nM) range [32]. At these levels in vivo and in vitro measurement of zinc necessitate use of highly specific and sensitive zinc chelating probes and zinc binding protein sensors. However, the biophysical properties of these compounds often limits their ability to discriminate biologically important transitions within intracellular zinc pools [32].
Distinguishing these changes is critical to reveal the metabolic trafficking and storage mechanisms that regulate subcellular zinc flux and largely determine the ultimate physiological impact of zinc.

**Cellular Zinc Metabolism**

The regulation of zinc metabolism takes place at the cellular level. It requires integration of signaling, membrane transport and protein sequestration in concert with changes in transcription, translation, post-translational modification and trafficking. Protein bound zinc, characteristic of that bound to zinc fingers, is avidly bound with high affinity. Consequently, changes in intracellular labile zinc do not directly influence DNA binding and activity of the vast majority of zinc containing transcription factors. However, transient changes in cytosolic zinc do impact gene transcription directly through metal-response element binding transcription factor-1 (MTF-1) and secondarily through interactions with kinases and phosphatases thereby influencing transcription as a second messenger [21, 33]. Zinc can also control production of other elements that regulate transcription [34]. MTF-1 contains six zinc finger motifs, two of which have lower than normal affinities for zinc and act as a sensor of intracellular zinc change by loosely binding zinc ions, leading to nuclear localization and DNA binding [35]. MTF-1 regulates transcription by binding to metal responsive elements (MREs) in the promoter regions of genes that regulate antioxidant and zinc homeostasis including metallothioneins (MTs). MT
expression is induced by zinc through MTF-1 and thus serves an indicator of increased cytosolic zinc [36]. MTs are a family of intracellular zinc binding proteins that sequester up to seven zinc ions when present at nanomolar (nM) and pM concentrations. MTs buffer intracellular labile zinc concentrations, acting as an exchangeable pool that accounts for approximately 15% of total intracellular zinc [37, 38]. The reactive oxygen species (ROS) sensitive transcription factor nuclear factor, erythroid 2 like 2 (Nrf-2) also up-regulates MTs [39]. MT-bound zinc is released from MT sulfhydryl groups in response to increased levels of ROS and reactive nitrogen species (RNS) [40]. Following zinc release, MT thiol groups are free to scavenge free radicals or to bind cytotoxic heavy metals like cadmium. Meanwhile, the newly liberated zinc ions may bind other proteins and function as ancillary antioxidants or second messengers. Alternatively those zinc ions may bind and activate MTF-1 potentially inducing production of additional MT [41]. Accordingly, MTs act as zinc and/or ROS/RNS induced intracellular zinc reservoirs during oxidative stress and function as a detoxification mechanism linking zinc metabolism to redox biology.

MTs buffer cytosolic zinc in order to maintain labile concentrations in the pM to low nM range, which is complimented by compartmentalization of zinc into or out of the cytoplasm or vesicles and organelles. This trafficking is accomplished primarily by 24 known dedicated zinc transport proteins (Fig.1). In addition to cellular distribution these transporters also control the bodily distribution of zinc. Although they display functional redundancy each has distinct
induction patterns, expression profiles, subcellular localization and tissue distribution, providing each transporter a unique role in zinc metabolism. Ten solute carrier 30A (SLC30A) family member zinc transport proteins (ZnTs) remove zinc from the cytoplasm across the plasma membrane or into cytosolic organelles. Conversely, fourteen solute carrier 39A (SLC39A) family members the Zrt-Irt-like-Proteins (ZIPs) move zinc into the cytoplasm from the extracellular environment or out of intracellular vesicles [42]. Individually, some ZIPs and ZnT proteins have been shown to traffic other divalent cations [43-45]. Further, other nondedicated divalent cation transporters have the capacity to transport zinc including natural resistance-associated macrophage protein 1 (NRAMP1), divalent metal transporter 1 (DMT1) and ferroportin 1 [46-48].

All ZnTs are predicted to have cytoplasmic amino and carboxy termini with six trans-membrane domains with the exception of ZnT5 which has 15 [49]. They function as zinc / hydrogen (H+) exchangers that exploit vesicular proton gradients [50] and affect intravesicular pH. ZnT subcellular location and function is altered by homo- and heterodimerization interactions [51]. ZnT1 is zinc responsive and regulated through activation of MTF-1 [52]. Zinc induces its expression through MTF-1 in murine cells [53] and zinc deficiency down regulates its expression in murine peripheral blood mononuclear cells (PBMCs) [54]. In human macrophages M.tb infection induces MTF-1 nuclear localization and ZnT1 expression [55] and LPS induces ZnT1 expression in murine dendritic cells in a TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent
manner [56]. However, exposure of primary human monocytes to LPS reduces ZnT1 expression indicating that it is differentially regulated by immunoregulatory transcription factors [57]. ZnT1 is ubiquitously expressed in tissue and localizes to intracellular vesicles as a heterodimer with ZnT3 and/or the plasma membrane as a homodimer or a heterodimer with either ZnT2 or ZnT4 [51, 58]. In particular ZnT1 localizes to the basolateral membrane in polarized cells of the intestine [59] or the apical membrane in the pancreas [60]. This suggests it actively participates in transfer of zinc out of cells into vascular circulation. The closest homolog of ZnT1 is ZnT10 which is thought to transport both manganese [43] and zinc and is highly expressed in human liver, brain and intestine tissue [61]. ZnT10 expression is up-regulated in the human intestine by vitamin D supplementation, which was shown in a human cell line to be due to vitamin D-dependent VDR transcriptional activation at its promoter [62]. Conversely, its expression is decreased by interleukin 6 (IL-6) in neuronal cell lines [63] further illustrating the interdependence of nutrient metabolic pathways with one another and inflammation.

What follows is brief description of the other ZnT transporters (as reviewed by Cousins and Lichten as well as Kambe et. al) [42, 64, 65]. ZnT2 localizes to intracellular vesicles including lysosomes in secretory tissues where it presumably increases intravesicular zinc levels. Its expression is zinc responsive and regulated by hormones including prolactin, testosterone and glucocorticoids through signal transducer and activator of transcription (STAT)-5. ZnT3 is
expressed exclusively in the brain, on synaptic vesicles where it increases intravesicular zinc. ZnT4 (along with ZnT6) is up-regulated by LPS in murine dendritic cells. It has been localized to vesicular organelles and the plasma membrane and is also expressed in the mammary gland, prostate, brain and intestinal epithelia. ZnT5 has ubiquitous expression and heterodimerizes with ZnT6 to transport zinc into the trans-Golgi network (TGN) of the cellular secretory pathway resulting in activation of alkaline phosphatase. It is also up-regulated in the liver by LPS. Due to the lack of a conserved zinc-binding site, ZnT6 is thought only to modulate zinc transport by ZnT5. In the murine intestine ZnT7 is required for intestinal zinc absorption and also accumulates in the trans-Golgi network but minimally impacts phosphatase activation. ZnT8 localizes to the insulin storage granules of β–cells in pancreatic islets, providing zinc for crystallization with insulin that is essential to its secretion. In mice its expression is down regulated in response to inflammatory cytokines tumor necrosis factor alpha (TNFα) and interleukin 1 beta (IL-1β). Single-nucleotide polymorphisms (SNPs) in SLC30A8 are associated with reduced insulin secretion; the gene is a major risk locus for type 2 diabetes. Further, ZnT8 is a major auto antigen implicated in type 1 diabetes. ZnT9 is a misnomer, it is actually a component of the p160 transcriptional coactivator complex that possesses a ZnT homologous cation efflux domain, it has been renamed GAC63.

The 14 ZIP cytosolic zinc import proteins are predicted to have 8 membrane-spanning domains and extracellular or intravesicular amino and
carboxy termini. They are thought to homo- or heterodimerize with themselves or other ZIPs. ZIP8 was discovered due to its induction in human monocytes in response to exposure to inflammatory stimuli including *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) cell wall cytoskeletal extract, LPS, TNFα and BCG infection [66]. In human monocytes, ZIP8 is expressed following LPS-dependent nuclear localization and transcriptional activation by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [33]. In human chondrocytes micro-RNA 488 selectively targets ZIP8 mRNA [67]. ZIP8 has multiple glycosylation sights [68-70], which may influence its membrane orientation, localization and binding partners. Transport by ZIP8 is pH dependent with optimal activity around pH 7.5 and extracellular bicarbonate levels increase its activity. It is also electroneutral, indicating it facilitates cotransport of other ionic species [69, 71]. ZIP8 is present on the plasma membrane and on intracellular vesicles and organelles including mitochondria, early endosomes and lysosomes in human cell lines, macrophages, upper airway epithelial cells and T-cells [30, 33, 66, 68, 71], indicating a role for ZIP8 in cellular zinc influx and the phagocytic pathway. ZIP8 causes lysosomal efflux in T-cells [30] and increases intracellular zinc concentrations in cell lines [33, 66]. Viral overexpression of ZIP8 in murine chondrocytes increases MTF-1 nuclear localization and transcriptional activity [72]. Furthermore, SLC39A8 hypomorphic mouse fetal fibroblasts have reduced MT expression in response to TNFα [33], indicating that ZIP8-dependent zinc possibly increases zinc-induced MTF-1
transcription of MT. ZIP8 expression is not altered by murine zinc status [54], but there is evidence in cell lines to indicate that intracellular zinc depletion increases ZIP8 levels on the plasma membrane [33]. ZIP8 also participates in cytosolic influx of manganese, iron, cadmium and selenite. Iron and zinc inhibit the ZIP8-mediated uptake of one another [69, 71, 73]. ZIP8 is most highly expressed in the human pancreas, lung, placenta, liver and thymus [66]. Its expression increases substantially in tissues during systemic inflammation in mice [74]. ZIP8 is also elevated in human PBMCs during sepsis [75] and in the circulating monocytes of HIV patients with acute viremia [76] as well as in locally inflamed tissues such as cartilage in osteoarthritis patients [72] and in the lungs of chronic smokers [77]. ZIP8 activity subsequently modulates the inflammatory response [30, 33, 72]. Finally, several rare genetic diseases result from nonsynonymous SNPs in the SLC39A8 gene that lead to dysfunctional zinc [78] and manganese [78, 79] metabolism.

ZIP14 is the closest paralog of ZIP8 and shares many of its characteristics [45]. For example, it is also responsive to inflammatory stimuli [80, 81] and transports zinc, cadmium, iron, and manganese [82]. Further it localizes to the plasma membrane, endosomes and lysosomes and is optimally active at a pH of approximately 7.5 with electroneutral transport that is elevated by bicarbonate [70]. It is most highly expressed in the liver at levels 10x higher than ZIP8 as well as in the pancreas, heart [83] and intestine [84]. In response to inflammation ZIP14 but not ZIP8 is induced in hepatocytes, which redistributes vascular zinc
into the liver [81]. Due to their differential tissue distribution and cellular expression, ZIP14 and ZIP8 have nonredundant functions and distinct physiological impacts. ZIP2 is present at the plasma membrane in cell lines where it increases cellular uptake of zinc [85]. In mice its expression is low and limited mostly to the skin, liver and tissues of the reproductive tract [86]. It is also expressed in human monocyctic cells where it is significantly increased by zinc chelation in a line of monocyctic leukemia (THP1) cells [87]. In murine macrophages it is up-regulated in response to activation by GM-CSF, elevating intracellular zinc concentrations and MT transcription [88].

Below is a consolidated description of the other ZIP transporters (as reviewed by Lichten and Cousins, Kambe et al. as well as Jeong and Eide) [42, 64, 89]. ZIP1 is ubiquitously expressed in human tissue and is found on the plasma membrane during low zinc conditions and is endocytosed to intracellular organelles in high zinc conditions. Its expression is increased in response to hormones but is insensitive to zinc concentrations in vivo or in vitro. ZIP3 is expressed mostly in the testis where it transports zinc into cells across the plasma membrane but is redistributed to intracellular organelles with increased zinc concentrations. ZIP4 transports zinc across the apical membrane into enterocytes in the small intestine and is responsible for the majority of human zinc absorption. It has a number of clinically significant mutations, which result in zinc malabsorption. Its expression is up-regulated through the transcription factor KLF4 in response to low zinc concentrations and it is endocytosed and/or
degraded in response to increased intestinal zinc levels. ZIP5 is constitutively expressed in polarized enterocytes and pancreatic acinar cells and is localized to their basolateral membrane. In low zinc concentrations its translation is decreased and it is internalized. ZIP6 expression is induced by estrogen and is a marker for estrogen-receptor positive cancers where its importation of zinc across the plasma membrane is associated with increased invasive metastatic progression. It is also LPS responsive and important in dendritic cell (DC) maturation where it negatively regulates major histocompatibility class II (MHCII) surface expression. ZIP7 localizes to the endoplasmic reticulum (ER) and the TGN where it is activated by phosphorylation. After activation it releases zinc that increases kinase activity of protein kinase B (AKT) and extracellular signal-regulated kinases 1 and 2 (ERK) and thereby migration and proliferation. ZIP9 localizes to the plasma membrane and the TGN where the zinc it transports from the TGN activates AKT and ERK. ZIP10 is essential for B cell survival and function; it is localized to the plasma membrane and its expression in mice is induced through MTF-1, leading to up-regulation in low zinc conditions and suppression when zinc is elevated. ZIP11 is localized to the nucleus and TGN in the testes and intestinal tract where its expression is not altered by zinc status. ZIP12 transports zinc across the plasma membrane and it is selectively expressed in the central nervous system (CNS) where it functions in neuronal differentiation and function. ZIP13 is localized to intracellular organelles where it
functions to transport zinc into the cytosol thereby altering cellular signaling events that effect connective tissue development in mice.

In summary zinc trafficking through both ZIP and ZnT transporters is critical in regulating the balance of zinc between protein bound, vesicular and labile pools. Each transporter has a unique and essential function that is based upon its expression, structure, tissue distribution, and subcellular location. In order to function effectively this refined system requires adequate zinc. Although the system is evolved to adjust to reduced zinc availability, prolonged deficiency can lead to deficits beyond the capacity of counter regulatory measures. Prolonged zinc malnutrition alters human zinc metabolism to conserve bodily zinc content and has many deleterious effects. Pathological disturbances that disrupt zinc absorption and excretion can also induce zinc deficiency or insufficiency. Thereafter, supplementation strategies rely on zinc metabolic processes to restore homeostatic concentrations and zinc dependent bodily function.

**Zinc Deficiency**

The requirement of dietary zinc intake in humans was first recognized during clinical investigations into growth and developmental deficiencies of an unknown etiology, which were eventually determined to be due to zinc deficiency [90]. Zinc deficiency occurs on a spectrum where moderate deficiency causes less extreme but consequential physiological disruptions similar to those manifested in severe zinc deficiency, which if left untreated is lethal. Zinc
deficiency is associated with increased susceptibility to infection especially in vulnerable populations. For example, zinc deficiency is associated with increased incidence of respiratory infections in the elderly [91] and children [92]. Currently there are an estimated 2 billion people suffering from zinc deficiency, [93] primarily in the developing world. Zinc deficiency is responsible for over one hundred thousand childhood deaths annually [94]. In addition to growth and development, adequate zinc nutrition is now known to be essential for neurological and sensory processing, stress tolerance and immunity [93]. Disease phenotypes associated with zinc deficiency result from impairment of zinc’s cellular functions including but not limited to: regulation of growth, coordination of cellular proliferation and apoptosis, prevention of tissue injury though detoxification of redox and peroxidation reactions as well as modulation of essential immunological host defense responses [4]. The fact that zinc is a type II nutrient and therefore does not elicit indicative clinical signs makes diagnosis of insufficiency and deficiency problematic. Type II nutrient deficiency responses have evolved to maintain tissue nutrient levels. The human bodies response to zinc deficiency leads to conservation of zinc through increased absorption and decreased excretion in urine and feces, coupled to growth inhibition and weight loss [31]. As with other nutrients, zinc deficiency may arise for several reasons the most widespread of which is insufficient dietary intake. Global economic disparity provides the substrate for food scarcity and in the extreme, famine. General unavailability of food results in malnutrition with the co-
occurrence of multiple nutrient deficiencies, including zinc deficiency with a cumulatively deleterious impact on health.

Zinc deficiency is also prevalent in disadvantaged populations that are not in a state of starvation [93]. Since foods rich in bioavailable zinc such as red meats are relatively expensive, they are reserved for more affluent populations whereas zinc poor foods and foods high in zinc-binding phytic acids like grains and legumes are more readily available to the economically disenfranchised. All seeds including grains contain phytic acids that bind divalent cations to form phytates, thereby sequestering zinc and making it unavailable for absorption [95, 96]. So although meat and grain centered diets may both contain equimolar zinc concentrations, diets with high levels of phytates will prevent zinc absorption leading to zinc deficiency in the absence of general malnutrition. There is also an anthropological explanation for the apparent disparity between zinc requirements and the absorbable zinc content of foodstuffs. The diet of the Paleolithic hunter-gatherer lifestyle in which humans evolved was rich in sources of bioavailable zinc like meat, fresh fruits and vegetables. However, transition to agricultural cultivation and processing of grains simultaneously introduced phytates and zinc poor foods and increased population to the point where zinc rich foods became relatively scarce [97]. Compounding the problem, zinc poor or high phytate diets have become culturally ingrained in some populations unintentionally leading to deep-seated advocacy of low zinc, high phytate foods. Additionally, pharmaceutical chelators such as penicillamine and diethylenetriamine
pentaacetate (DTPA) as well as ethambutol and tetracycline antibiotics can cause secondary zinc deficiency by similarly binding and thereby reducing bioavailability and absorption of zinc [98]. Fortunately, increased zinc intake through dietary adequacy, zinc supplementation and zinc-fortified foods can alleviate and often lead to complete resolution of many of the ill effects of zinc deficiency [99] if spaced appropriately from zinc sequestering compounds.

Malabsorption disorders can also increase the risk of zinc deficiency, particularly in diets that provide marginal zinc nutrition. Inflammatory enteropathology causes zinc malabsorption and deficiency due to the decreased absorptive capacity associated with disturbed intestinal architecture. Populations subjugated to poverty and in particular children, are disproportionately predisposed to malabsorptive zinc deficiency associated with environmental enteropathy (EE) [100]. Poor sanitation practices linked to lack of infrastructure and education lead to recurrent fecal ingestion causing the chronic intestinal inflammation and subsequent histopathological changes that characterize EE [101]. Patients with other inflammatory enteropathologies including Crohn’s disease [102] or celiac disease [103] are also predisposed to zinc deficiency due to impaired absorption. Interestingly zinc supplementation improves intestinal barrier function and reduces the pathological permeability associated with Crohn’s disease [104]. Conversely, institution of a gluten free diet alone reduces inflammation and normalizes zinc levels in celiac patients [103]. Conditions that shorten the intestinal absorptive surface such as short bowel syndrome [105] or
intestinal bypass surgery [106] can also result in inadequate zinc absorption and require zinc supplementation. The rare autosomal inherited genetic zinc malabsorption disorder acrodermatitis enteropathica (AE) [107] results from phenotypic expression of defects that arise from a number of mutations of ZIP4 [108]. This condition prevents effective intestinal zinc absorption and disrupts metabolic control of zinc distribution. It results in severe zinc deficiency and is fatal without treatment with high dose zinc supplementation. AE clearly outlines the sequelae associated with zinc deficiency including immune dysfunction and increased mortality due to infections. Diseases that increase zinc loss through excretion can also cause zinc insufficiency or deficiency. Cirrhosis, which is often associated with chronic alcoholism and viral infection, reduces circulating albumin levels impairing serum zinc binding capacity and leading to increased renal zinc excretion and deficiency [109]. Similarly diabetes patients exhibit hyperzincuria [110], reduced serum zinc concentrations [111] and ubiquitous cellular zinc depletion [112] compared to healthy controls. In light of the association between ZnT8-dependent zinc transport dysfunction and diabetes [65] these findings demonstrate how dysfunctions in cellular zinc metabolism in seemingly unrelated systems can indirectly result in zinc deficiency.

Infection can also contribute to zinc deficiency. HIV infection reduces the intake, absorption and metabolism of zinc [113]. However, although reduced serum zinc concentrations are often observed in HIV they are also associated with an increase in monocyte ZIP8 expression and subsequent cellular
internalization of zinc [76]. Hence, zinc levels in HIV positive subjects are not a reliable measure of zinc status. HIV is associated with excessive diarrhea, which increases zinc losses and increases dietary requirements. Infectious diarrhea, both viral and bacterial, is a major cause of childhood morbidity and mortality in the developing world and a major communicable cause of zinc deficiency [114]. Infection is a driving force in the pathological intestinal remodeling of EE that results in zinc deficiency [100, 101]. Conversely zinc deficiency impairs immunity increasing susceptibility to infectious diarrhea [115]. The incidence, severity and duration of diarrhea are reduced by zinc supplementation [92, 116]. Most importantly, zinc supplementation has shown to significantly reduce diarrhea associated hospital admission and mortality in combination with the standard of care [117]. Accordingly, therapeutic zinc supplementation is now a standard component of global diarrhea control [118].
1.4. Nutrient Dependent Host Defense

Malnutrition and Immunity

The immune system is the fundamental protective network of humans that consists of anatomical structures, specialized cells and protective protein constituents that cumulatively maintain resistance to pathogens. It is comprised of the innate and adaptive systems. The innate immune system is responsible for immediate protection and relies upon recognition of conserved pathogenic features to trigger immune activation and combat infection. It is comprised of physical barriers including the skin and mucosa, the components of complement and specialized sets of cells. The dedicated cells of the innate immune system include: myeloid lineage monocytes, dendritic cells (DCs), macrophages, granulocytes (neutrophils, basophils and eosinophils) and mast cells as well as lymphoid derived natural killer (NK) cells. A finite collection of pathogen associated molecular patterns (PAMPs) activates sets of dedicated extracellular and intracellular receptors to initiate the innate immune response. PAMP recognition triggers distinct arrays of host defense responses for each cell type through activation of complex, interdependent, intracellular signaling networks.

Professional phagocytic cells including monocytes (bloodstream), macrophages (tissue) and neutrophils (bloodstream) are evolved to respond to PAMP recognition with cytoskeletal rearrangements that initiate ingestion of the offending pathogen through phagocytosis. In most cases there is subsequent intracellular killing of microbes through ROS generation, acidification and
trafficking of the newly formed phagosome to fuse with the lysosome for enzymatic degradation. In the case of monocytes, macrophages and DCs the digested microbial products of the lysosome can then be presented as antigens to cells of the adaptive immune system. Pathogen recognition by cellular receptors such as the Toll-like receptors (TLRs) initiates release of soluble mediators of inflammation. Monocytes and macrophages initiate and propagate inflammation in response to PAMP recognition in part through the release of pro-inflammatory cytokines including but not limited to TNFα, IL-1β, IL-6 and chemokine interleukin 8 (IL-8). Those same cells also temper inflammation with immunomodulatory cytokines such as interleukin 10 (IL-10). Inflammatory mediators and chemokines help to recruit and activate additional innate immune cells as well as cells of the adaptive immune system.

The adaptive immune system constitutes a more efficient but delayed mechanism of defense, characterized by the ability to assimilate the recognition of new antigenic patterns through prior exposure. Lymphoid cells of the adaptive immune system include distinct sets of T cells that coordinate and carry out cellular immunity and B cell subsets that produce antibodies of the humoral response. Antigenic exposure activates adaptive T cell responses including release of cytokines such as interferon gamma (IFN-γ), which attracts macrophages and activates their antimicrobial capacity and interleukin 2 (IL-2), which aids in lymphocyte differentiation. Lymphocyte differentiation in response to antigen presentation produces effector cells that act to eliminate the current
infection and memory cells that prepare the host for immediate action against subsequent reinfections. In this way the adaptive immune system provides specific long lasting protection against reoffending pathogens.

Nutrition has long been recognized as a central component of health and a fundamental aspect of disease prevention and therapy [119]. In general, severe calorie restriction due to macronutrient malnutrition reduces health and drives immune dysregulation. It leads to atrophy of the thymus and reduced circulating lymphocyte numbers [120]. Protein energy malnutrition (PEM) specifically is associated with impaired barrier function and impaired cellular host immunity, particularly lymphocyte function [121] and cytokine dysregulation [122]. Malnutrition drives immunity toward innate immune responses due in part to their comparatively more efficient energetic demands. However, prolonged nutrient deprivation also disrupts myeloid cell function [123], cumulatively leading to increased susceptibility to infectious disease [124]. Likewise, comorbid infection leads to increased production of pro-inflammatory cytokines [125, 126], which suppress appetite and further exacerbate malnutrition and dehydration. Conversely, over-nutrition also exacerbates inflammatory cytokine release [127, 128], reinforcing the principal that proper nutrient balance is essential in maintaining optimal immune function. Additionally, deficiencies of macrominerals and micronutrients can disrupt critical immune functions evolved to utilize the specific biophysical and chemical characteristics of individual nutrients toward host defense.
Among micronutrient deficiencies, zinc deficiency is particularly difficult to identify as a cause of immune impairment. Due to its unique position in human health and broad essentiality, zinc deficiency mirrors (and may be responsible for) many of the immune dysfunctions of macronutrient malnutrition. Zinc deficient murine models display lymphopenia and thymic atrophy [129] with reduced ability to generate T cell [130] and B cell [131] responses which is due to increased apoptosis of precursor cells [132]. Further because zinc is a necessary cofactor of the T cell differentiation hormone thymulin, zinc deficiency reduces its bioactivity [133, 134] in addition to its production, thereby reducing lymphocyte maturation. Human experimental models reveal that even mild zinc deficiency reduces thymulin bioactivity and T cell maturation [134]. AE patients present with derangements in lymphocyte maturation which is resolved with zinc supplementation [135]. Zinc supplementation increases thymus size in patients recovering from PEM [136] indicating that the atrophy and thymic dysfunction associated with general malnutrition may in fact be due to zinc deficiency. Lymphopenia due to zinc deficiency in humans causes immune imbalance characterized by reduced natural killer (NK) cell lytic activity and a decrease in the ratio of CD4$^+$ to CD8$^+$, cells which is corrected with zinc supplementation [137]. Zinc supplementation of deficient children increases the CD4$^+$ to CD8$^+$ ratio as well as IFN-$\gamma$ and IL-2 release from PBMCs stimulated with PHA [138]. Human experimental models of moderate zinc deficiency also show reduced production of IL-2, and IFN-$\gamma$ in response to phytohaemagglutinin (PHA) which is
corrected with supplementation [137]. Additionally, following prolonged culture in zinc deficient conditions both IL-2 and IFN-γ expression is reduced in human T cell lines exposed to phorbol 12-myristate 13-acetate (PMA) and PHA or PMA and Ionomycin, respectively [139]. Likewise in human whole blood prolonged zinc supplementation in culture increases PHA induced IFN-γ release [140]. These results indicate that zinc deficiency inhibits Th1 cell cytokine responses thereby reducing cellular adaptive immunity and subsequent activation of monocytes and macrophages.

In comparison to the negative impact of zinc deficiency on lymphocyte numbers, myeloid populations in murine models are maintained, however, zinc deficiency also negatively impacts function of the innate immune response [141]. Although total numbers of phagocytes do not decrease during zinc deficiency in human patients, the chemotactic responsiveness of monocytes and neutrophils is reduced and can be restored by zinc supplementation [142, 143]. Human zinc deficiency has also been shown to impair neutrophil ROS-dependent host defense [144]. Zinc supplementation following inflammatory stimulation increases the oxidative burst in human neutrophils [145], due to zinc-dependent activation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [146]. Zinc supplementation also increases the phagocytic capacity of human neutrophils and monocytes [147] as well as murine macrophages [148] for bacteria. Zinc deficiency in mice reduces macrophage phagocytosis and killing of Trypanosoma
cruzi and cellular supplementation of zinc, but not copper manganese or nickel, restores their phagocytic antimicrobial capabilities [149].

In addition to reduced recruitment and phagocytosis, zinc deficiency and supplementation have dramatic effects on monocyte and macrophage inflammation in response to PAMP recognition and infection. The impact of zinc on monocytic cell inflammatory cytokine production is variable depending upon in vitro model. Some in vitro studies have shown that zinc deficiency reduces proinflammatory cytokine release and implicate zinc supplementation as an enhancer of inflammation. In human PBMCs acute zinc deficiency induced by the membrane permeable zinc specific zinc chelator N,N',N',N-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN) reduces production of TNFα during infection with Escherichia coli (E.coli) or Staphylococcus aureus (S.aureus) and reduces TNFα and IL-6 production following exposure to LPS [150]. Similarly, in the human monocytic cell line Mono Mac1 TPEN pre-incubation completely eliminated LPS-induced release of TNFα, IL-1β and IL-6 where pre-incubation with zinc and the membrane permeable zinc trafficking ionophore pyrithione increased their release [151]. In human PBMCs release of pro-inflammatory cytokines IL-1β and TNFα is induced by super-physiological doses of zinc alone [152] or by concurrent exposure to LPS in the presence of physiological zinc doses [153]. Other in vitro studies have shown a pro-inflammatory effect of zinc deficiency with an associated anti-inflammatory effect of zinc supplementation. In the human monocytic cell line HL-60, prolonged culture in zinc deficient media
prior to PMA exposure increased TNFα, IL-1β and IL-8 mRNA expression [139] and TNFα and IL-1β release [34]. Cell lines pretreated with TPEN before exposure to PMA also released elevated levels of TNFα and IL-1β [154]. Pre-incubation of primary human monocytes with supra-physiological zinc concentrations prior to LPS exposure reduces TNFα release and co-incubation of zinc/pyrithione with LPS eliminates both TNFα and IL-1β production and release in that model [155]. Further, LPS-treated PBMCs derived from zinc-supplemented human subjects have reduced TNFα and IL-1β mRNA expression compared to LPS exposed PBMCs from placebo controls [19].

In comparison to disparate *in vitro* observations, zinc deficiency clearly elicits a hyper-inflammatory phenotype in *in vivo* murine models of sepsis. In sepsis, dysregulated hyper-inflammatory cytokine signaling primarily by monocytic cells disrupts the coordination of innate and adaptive host defense responses, reducing pathogen clearance and increasing morbidity and mortality. Zinc deficiency disrupts immune signaling during the acute phase response to infection resulting in an increased cytokine storm and sepsis. In murine models acute zinc deficiency induced by TPEN injection following a septic dose of LPS increases circulating levels of IL-1β and IL-6 [156]. Zinc supplementation of mice prior to exposure to LPS reduces TNFα production and liver injury [157]. Cecal ligation and puncture (CLP) induces polymicrobial infection and sepsis in mice [158]. In mice, moderate zinc deficiency established through dietary restriction of zinc intake leads to a reduction in circulating zinc levels to approximately half of
normal and induces elevations in circulating cytokines including IL-6, IL-10, IL-1β and chemokine CCL3 following CLP. These changes are associated with increased tissue destruction and cell death in vital organs as well as dramatically increased mortality that is partially rescued by zinc supplementation prior to CLP [159]. In that model, zinc deficiency increases bacterial burden in tandem with increased pulmonary expression of TNFα, IL-1β and the acute phase protein serum amyloid A (SAA) as well as circulating SAA protein [160]. Human zinc supplementation has shown to have variable effects on circulating cytokines [161]. However in human sepsis patients lower serum zinc concentrations are correlated with increased severity of illness [75] and mortality [162]. The fundamental causes that underlie zinc deficiency-associated alterations in immune function and susceptibility to disease are dysfunctions in the mechanisms of host defense that rely on zinc. Zinc metabolism and immunity are inseparable and interdependent. Zinc is utilized for host defense due to its unique biophysical profile, which among other actions allows it to regulate intracellular signaling through protein binding and to directly regulate microbial growth and survival by modulating its abundance.
Pathogen-Induced Intracellular Cationic Signaling

Pathogen recognition by innate immune cells is a fundamental first step in immune activation. It results in inflammation, antimicrobial killing and activation of the lymphocyte-dependent adaptive immune system through secondary presentation of microbial antigens [163]. During signaling dietary divalent cations are used as second messengers to coordinate aspects of this response by influencing the phosphorylation state of signaling proteins and cellular electrophysiology. Cells of the immune system have a negative intracellular membrane potential ($V_m$). Divalent cation transporters exploit this electrochemical gradient to facilitate cytosolic flux. Membrane depolarization is an important pathogen sensing response in cells of the immune system that is accomplished through dedicated ion channels. Receptor-mediated membrane depolarization is a function of calcium via store operated calcium release in lymphocytes. Membrane potential is maintained and rectified by voltage-gated channels responsible for potassium, sodium and chloride flux. [7]. Furthermore, lymphocyte activation is regulated by increased levels of intracellular calcium through the action of calcium-binding protein calmodulin on CaM kinases and phosphatases [164]. As such macronutrients are intricate to divalent cation signaling which is a critical initial host defense response to pathogen recognition.

Iron signaling also impacts host defense by regulating of the expression of inducible nitric oxide synthase (iNOS) and the production of the free radical signaling molecule (NO). Iron-dependent inhibition of DNA binding by

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CCAAT/enhancer-binding protein beta (C/EBPβ) at the promoter of iNOS reduces its expression [165]. Conversely, in murine macrophages, iron transported through NRAMP1 inhibits production of IL-10 thereby elevating iNOS expression resulting in subsequent increases in NO as well as TNFα and IL-6 and enhancing bacterial killing [166, 167]. It remains unknown if NRAMP1-dependent iron import modulates C/EBPβ, however IL-10 transcription is C/EBPβ-dependent [168], which may help to explain the impact of NRAMP1 on IL-10 production.

Modulation of zinc metabolism is among the most finely tuned and sensitive mechanisms regulating intracellular signaling. Cellular fluctuations in the intracellular pool of labile cytosolic or nuclear zinc determine its role as a second messenger. Zinc flux is controlled by ZIP and ZnT transporters as well as binding proteins such as calprotectin and MT. The expression, localization and activity profiles of these zinc regulatory factors can be altered in response to pathogen recognition, inflammatory signaling, ROS or intracellular zinc [42]. Cytosolic zinc signals can be categorized as fast signals occurring within seconds to minutes and slow signals that occur within hours to days following a given stimulus [169]. Fast signals can result from activation of zinc transporters [170] whereas slow signals often rely on changes in mRNA expression that result in new protein formation [30, 33]. Regardless of time scale zinc signaling is used to transmit information to and within cells and in particular to modulate the response of immune cells to potential threats. Accordingly, pathological
alterations of zinc status can disrupt the immune system’s ability to appropriately enact critical host defense functions through perturbation of cytokine signaling. Zinc can modulate immune signaling pathways by stabilizing signaling protein complexes, inactivating signaling enzymes, particularly kinases and phosphatases and also by modulating transcription factor binding.

Zinc influences T-cell signaling though multiple pathways. It induces T cell signal transduction by stimulating activation and membrane localization of protein kinase C (PKC) [171] and induces nuclear translocation and DNA binding of the transcription factor Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [172]. It also modulates T cell receptor (TCR) signaling by increasing Src-kinase Lck homodimerization, activation [173] and binding [174] but not recruitment to CD4 and CD8 [175]. TCR activation stimulates a fast zinc signal through ZIP6 that enhances calcium influx and proliferative responses in primary human T cells [175]. Further, human T cell activation induces expression and production of ZIP8 protein, which localizes to the lysosome and produces a subsequent slow zinc wave. ZIP8-dependent zinc then inhibits the phosphatase calcineurin resulting in increased phosphorylation and activity of the transcription factor cAMP response element-binding protein (CREB), thereby inducing IFN-γ expression [30]. This mechanism explains the necessity of zinc sufficiency for IFN-γ production. It is a clear illustration of how zinc signaling regulates inflammation and host defense in lymphocytes with repercussions in the innate immune system where macrophage activation relies on IFN-γ.
Zinc-dependent modulation of inflammatory signaling by monocytes and macrophages is among the most important mechanisms of micronutrient immunological regulation. In human monocyte-derived-macrophages (MDMs) activation of the intracellular PAMP receptor nucleotide-binding oligomerization domain-containing protein 2 (NOD2) increases intracellular zinc levels, leading to MTF-1 dependent MT production, which enhances zinc retention and bacterial clearance [176]. In human monocytes, exposure to LPS activates TLR4, generating a fast zinc signal [33, 151] and also inducing MT expression [177]. Zinc modulates NF-κB activation in monocytes and macrophages responding to LPS and E.coli. NF-κB is a critical mediator of inflammation and a central signaling axis of the innate immune system. It controls the immune response on many levels and is responsible in part for inducing transcription of cytokines including TNFα, IL-1β, IL-6 and IL-10 in response to pathogen recognition [178]. Alteration of NF-κB activity by zinc is a potential mechanism for the zinc-dependent cytokine dysfunction observed in zinc deficiency and corrected through zinc supplementation. Accordingly, increased NF-κB transcription induces pro-inflammatory cytokine release whereas decreased NF-κB activity reduces those cytokines. NF-κB consists of proteins including p65, p50, p52, RelB and c-Rel that can hetero- or homodimerize and are sequestered outside of the nucleus in an inactive state by IκB binding proteins. Activation of signaling cascades results in phosphorylation and degradation of the IκBs and subsequent NF-κB nuclear translocation [178]. There are multiple receptors and signaling
networks that regulate NF-κB activation and predictably zinc modulates numerous constituents of that activation.

The impact of zinc on NF-κB in vitro varies and is dependent upon the experimental conditions studied. Intracellular zinc deprivation by TPEN reduces phosphorylation of kinases responsible for activating NF-κB. Those kinases include: mitogen activated protein kinases (MAPKs) p38, MEK and ERK in E.coli infected Mono Mac 1 cells as well as IκB kinase (IKK) in LPS exposed human monocytes. TPEN also inhibits DNA binding of NF-κB subunit p50 in LPS exposed human leukocytes [151]. Conversely, zinc reduces NF-κB activity in monocytic cells through several mechanisms. Zinc/pyrithione or high dose zinc inhibits phosphodiesterases (PDEs) [155]. Zinc interferes with PDE-dependent reduction of cyclic guanosine monophosphate (cGMP) and subsequent protein kinase A (PKA) activity that would normally lead to reduced IKK phosphorylation and thereby NF-κB activation [179]. Another proposed mechanism is zinc-dependent increase in the expression of A20 deubiquitinase (A20). A20 inactivates TNF receptor-associated Factor 6 (TRAF6) upstream of NF-κB preventing its activation. In cell lines exposed to PMA, prolonged culture in zinc deficient conditions decreased A20 expression thereby increasing NF-κB nuclear localization and activity. Furthermore, siRNA mediated A20 knockdown eliminated zinc associated decreases in TNFα release [34].

There is also ZIP8-dependent feedback modulation of NF-κB. Expression of ZIP8 is increased in the peripheral monocytes of critically ill infected patients, a
response unique among human zinc transporters. Its expression occurs in conjunction with a dramatic reduction in plasma zinc concentrations [75], indicating that ZIP8 plays a role in the redistribution of vascular zinc into mononuclear phagocytes in response to infection. Furthermore, in murine models of polymicrobial sepsis NF-κB DNA binding is elevated in zinc deficiency, which is associated with cytokine dysregulation [160]. In human monocytes, macrophages, and murine alveolar macrophages ZIP8 expression is induced through NF-κB in response to LPS and TNFα [33]. ZIP8-dependent zinc also inhibits LPS-induced NF-κB transcription by reducing IkB kinase (IKK) activity as well as ERK phosphorylation, which results in inhibition of TNFα, IL-1β, IL-6 and IL-8 release [33]. Furthermore, the inhibitory concentration IC₅₀ required to inhibit IKK is much closer to the physiological nM – pM range [33] and well below the μM range that was previously reported in LPS activated murine macrophages [180]. Others have proposed a similar role for ZIP14 in LPS exposed human macrophages. In that model, ZIP8 induction by LPS was an order of magnitude greater than that of ZIP14 but was not explored [181]. Cumulatively, these studies reveal immune signaling mechanisms that rely upon zinc to transmit critical immune modulatory host defense mechanisms within monocytic cells. Furthermore through ZIP8, zinc tempers innate and adaptive inflammatory responses, regulating the immune systems pathogen recognition and defense mechanisms based upon zinc status.
Anti-Microbial Trace Element Redistribution

In addition to humoral and cellular immunity, immunological nutrient redistribution constitutes a distinct third mechanism of human host defense. The term nutritional immunity was coined to describe the anti-microbial benefits associated with systemic vascular iron depletion [2]. However it has come to encompass both systemic and cellular nutrient deprivation of multiple trace elements including iron, manganese and zinc from extracellular or intracellular pathogens. In contrast innate immune cells, particularly macrophages, also redistribute iron, copper and zinc into phagosomes following phagocytosis of microbes. This hyper-concentration leads to intraphagosomal formation of toxic ROS and disruption of enzymatic activity due to iron displacement from sulfhydryl moieties [182]. These changes lead to microbial death or growth reduction.

Iron concentrations are precisely regulated within the human body; as such most circulating iron is not bioavailable to microbes who require it for growth and survival [183]. It is bound by hemoglobin within erythrocytes or splenic and hepatic macrophages. Alternatively iron is bound by the proteins transferrin and lactoferrin within and outside of cells and by ferritin intracellularly. Transferrin receptor on the macrophage plasma membrane binds transferrin-bound iron and absorbs the complex through endocytosis. Iron is then released from transferrin due to endosomal acidification and subsequently trafficked into the cytosol by DMT1 where it is stored in complex with ferritin [184]. Many bacterial and fungal pathogens have evolved high-affinity iron chelating siderophores capable of
capturing host protein bound iron. To counter this, neutrophil- and macrophage-derived small peptide lipocalin-2 sequesters iron laden siderophores away from bacteria [185]. Systemic iron redistribution in response to infection is controlled by the cationic antimicrobial peptide hepcidin [186]. Hepcidin is produced by the liver in response to high extracellular iron levels or elevation of IL-6 in response to infection by bacterial, fungal and viral pathogens [187]. Recognition of bacterial PAMPs also induces hepcidin expression in macrophages [188]. Hepcidin binds and targets the cellular iron exporter ferroportin for degradation [189]. Loss of ferroportin limits circulating iron concentrations by reducing dietary iron absorption and trapping iron inside of macrophages. Activation of macrophages by IFN-γ or LPS reduces ferroportin and transferrin receptor expression and increases transcription of the iron importer DMT1 [190] thereby prolonging systemic iron deprivation. IL-6 and IL-10 production in response to infection further reduces circulating iron levels by inducing CD163 expression in macrophages, which leads to uptake of hemoglobin-haptoglobin bound iron [191]. Cumulatively, this limits growth of iron dependent extracellular pathogens but can also induce anemia during chronic infection and inflammation [192].

A comparable redistribution of zinc occurs during the acute phase response. ZIP14 is a transporter of ferrous iron as well as zinc [82] and is induced by IL-6 in the liver of mice following LPS exposure [81]. The hypozincemia response is proposed to serve a similar role to iron restriction by reducing availability of extracellular zinc nutrition to invading pathogens. ZIP8
[33] and ZIP14 [181] induction by LPS in macrophages may contribute to this extracellular hypozincemia particularly within the local microenvironments of tissues. ZIP14 knockout mice do not exhibit this transient hypozincemia response and have reduced uptake of intestinal zinc [193]. ZIP14 is present on the basolateral membrane of enterocytes and functions to transfer dietary zinc into circulation [84], which may transiently increase zinc necessary for host defense during infection. Production and export of the zinc [194], manganese [195] and iron [196] binding protein dimer S100A8/S100A9, also known as calprotectin, is up-regulated in response to bacterial invasion. Calprotectin serves as an extracellular sink for these vital nutrients thereby preventing exploitation by pathogens [197]. It is primarily expressed in myeloid cells including neutrophils, monocytes and macrophages [198]. It is also an activator of TRL4 [199] and propagates the inflammatory response through NF-κB leading to up-regulation of proinflammatory cytokines [200]. Therefore calprotectin simultaneously reduces zinc availability and induces inflammation, which taken in the context of ZIP8-dependent zinc inhibition of NF-κB, may propagate hyper-inflammation, thereby linking zinc signaling and nutritional immunity. Importantly circulating levels of bioavailable zinc are also kept low through binding to α-2 microglobulin and transferrin [27]. However, some bacterial siderophores can bind zinc and may function to capture zinc in a strategy similar to iron acquisition [201].

Although intracellular nutrient sequestration may benefit the host against extracellular pathogens it has the potential to facilitate nutrient availability to
intracellular microbes. Depending on the individual properties of the nutrient, intracellular accumulation of zinc, iron, copper and manganese has variable effects on the growth and survival of intracellular pathogens. Accumulation of intracellular iron stores within macrophages increases iron availability to intracellular pathogens within macrophages and impairs IFN-γ-mediated macrophage TNFα production and antigen presentation [185]. Subsequently, in vitro and in vivo studies reveal that hepcidin-dependent iron overload in macrophages increases intracellular pathogen growth [202]. Furthermore, some intracellular pathogens are capable of capturing iron from exogenous and endogenous stores [203, 204]. Macrophages have evolved a complex system of intracellular iron redistribution to counter microbial exploitation of cellular iron internalization. The primary mechanism of defense is modulation of intraphagosomal iron content. The two transporters that are known to primarily control intraphagosomal iron redistribution in macrophages are NRAMP1 and ferroportin 1 [205, 206]. NRAMP1 is a proton/divalent cation antiporter [207] with broad substrate specificity including iron, zinc, copper and manganese [46]. It is capable of shuttling metals bi-directionally against a proton gradient whereby the direction of transport is determined by proton and divalent cation concentrations [207]. In murine models NRAMP1 is rapidly localized to the phagosome [206, 208] and is associated with resistance to intracellular pathogens [209] through a number of antimicrobial mechanisms. NRAMP1 actively acidifies the bacterial phagosome in mice [210]. It increases translocation of the proton ATPase to the
phagosome following IFN-γ activation leading to generation of Fenton-mediated free radical production [166, 167]. Iron can be imported into the phagosome through NRAMP1 in the setting of high pH and low iron intraphagosomal concentrations, resulting in generation of ROS through the Fenton and Haber-Weiss reactions [211, 212]. Alternatively, in instances where intraphagosomal concentrations are high, NRAMP1 can export iron and manganese out and import protons into the phagosome, increasing acidity and depriving pathogens of essential nutrients [167, 213]. Ferroportin 1 is also expressed on the phagosomal membrane of murine cells and may serve to sequester iron nutrition away from bacteria [205].

Macrophages also use a strategy of phagosomal hyper-concentration of copper [214] through a separate set of copper transporters [16]. IFN-γ activation of murine macrophages induces expression of the plasma membrane copper transporter CTR1 leading to copper uptake. Translocation of the copper importer ATP7A to the phagosome then increases intraphagosomal concentrations of copper and subsequently generation of bactericidal Fenton free radicals [215].

Zinc is an essential trace element necessary for the growth and survival of all prokaryotes [216]. Macrophages also manipulate levels of unbound cytoplasmic zinc in response to intracellular pathogens, resulting in reduced microbial growth and viability. Zinc is sequestered away from the fungal intracellular macrophage pathogen *Histoplasma capsulatum* (*H. capsulatum*) following granulocyte macrophage colony-stimulating factor (GM-CSF) exposure.
This leads to increased production of intracellular zinc binding species thereby limiting zinc availability to *H. capsulatum* and growth inhibition [88, 217]. Further, GM-CSF increases murine macrophage zinc uptake through up-regulation of ZIP2 in response to *H. capsulatum*. Resulting induction of MT expression then reduces zinc cytosolic availability, which is accompanied by zinc deprivation and increased ROS generation within the fungal phagosome [88]. A mechanism of zinc deprivation of the bacterial phagosome in macrophages and dendritic cells through up-regulation and trafficking of ZIP8 to the phagosome-lysosome pathway has also been proposed [218]. Alternatively, macrophages have been shown to super-concentrate zinc within the bacterial phagosome [214] and poison bacteria that reside within those phagosomes [55, 219]. Zinc hyper-accumulation in human macrophages reduces survival of *E.coli*. In contrast, zinc accumulation causes up-regulation of bacterial zinc efflux pumps critical for adaptation of intracellular pathogens including *Salmonella typhimurium* (*S.typhimurium*) and *M. tuberculosis* [55, 219]. Although no definitive mechanism for zinc-associated toxicity was demonstrated in these models there are several potential avenues through which elevated zinc concentrations may be toxic to pathogens. Those mechanisms include displacement of iron from sulfhydryl moieties of bacterial enzymes [182] or disruption of manganese uptake which reduces bacterial free radical tolerance [220]. Additionally, zinc redistribution into the bacterial phagosome was shown to be TLR-dependent [219] and to induce MTF-1 dependent up-regulation of ZnT1 and MT [55]. The authors went on to
hypothesize a model of ZnT phagosomal localization as the mechanism of zinc hyper-accumulation. However the potential for phagosomal NRAMP1 to transport zinc was not considered [46, 206]. Furthermore, MTF-1-dependent MT up-regulation [55] indicates that a cytosolic zinc signal was invoked to precipitate this response. In summary, macrophages use divalent cation metabolism in host defense against microbial invasion through modulation of inflammatory signaling and redistribution strategies. Macrophage micronutrient strategies have evolved to exploit the nutritional requirements of offending extracellular microbes. Additional studies regarding the interplay of nutrient acquisition between host and microbe are warranted for intracellular pathogens. Invasion of the intracellular space by microbes can lead to persistent infection through adaptation strategies employed by the pathogen. Understanding these complexities for the intracellular pathogen *M. tuberculosis* is particularly important given its substantial impact on global human health.
1.5. Tuberculosis

Subversion of human health by *Mycobacterium tuberculosis*

Tuberculosis (TB) is a global epidemic. It is a major cause of morbidity and mortality primarily in the developing world and is responsible for \(\sim 1.5\) million deaths each year. Along with HIV, which is predispositional to TB, it ranks as the leading cause of death worldwide. Globally it is estimated that 2-3 billion people are infected with *Mycobacterium tuberculosis* (*M.tb*) with over nine and a half million new infections annually. Five to fifteen percent of those infected will go on to develop active TB disease, primarily within the most economically productive age groups, between fifteen and fifty-nine years of age. Annually, an estimated 8 billion dollars is required to respond to the global epidemic [221].

Primary infection is established in the lungs following the inhalation of aerosolized respiratory droplets expelled from a contagious person. Initial infection of those with an intact immune system typically results in a latent, non-communicable, asymptomatic *M.tb* infection that may remain dormant or reemerge as an active, contagious, potentially fatal disease. The dose required for infection is estimated to be on average one bacterium. Upon inhalation, it travels through the respiratory tract into the distal alveolar space where it is phagocytosed by alveolar macrophages (AMs) [222]. The lung microenvironment is in general immunosuppressive and facilitates a growth permissive non-inflammatory phenotype in AMs [223]. Signals generated by infected AMs attract additional phagocytic cells including alveolar and interstitial macrophages and
DCs [224, 225]. The subsequently expanded number of infected cells eventually generates nascent granulomatous cellular lesions [226]. Infected DCs disseminate through the lymphatic system to thoracic lymph nodes [227]. There they present MHCII-bound mycobacterial antigens to CD4⁺ T cells thereby activating the adaptive immune response [228, 229]. Primed T cells are then recruited to the infected region where they secrete IFN-γ and TNFα to activate macrophage antimycobacterial host defense functions. These events help establish infection control, generating mature granulomas without fully eliminating *M.tb* [230]. Importantly it takes up to 4-6 weeks for adaptive immune activation [231, 232] and in that time infected macrophages remain permissive to rapid bacterial replication [223, 233]. Further, during that time bacteremia can occur leading to distant deposition of *M.tb* and formation of extrapulmonary granulomas [234]. *M.tb* can persist in an altered metabolic state confined within granulomas [235] for the life of an immune competent host or alternatively may reactivate into active TB following immune compromise and loss of granuloma integrity.

Individuals with chronically depressed immunity, including patients on long-term immunosuppressive therapy and those co-infected with HIV, have a much higher risk of developing TB disease. Because of this HIV and TB are considered co-epidemics with 400 thousand deaths due to TB in HIV-positive people annually [221]. Secondary immunodeficiency due to malnutrition is also a major risk factor for susceptibility to TB and is associated with increased incidence and severity [236]. Severe TB causes appetite suppression leading to
cachexia due to hyper-inflammation and blocks utilization of host nutrition for anabolic processes which compounds immunosuppression [237]. Further, underweight patients have an increased rate of relapse after treatment [238].

The curative treatment for TB consists of a prolonged regimen of multiple antibiotics with extensive side effects. However, lack of universal availability and poor patient compliance are impediments to effective global eradication. Poor compliance has increased the incidence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of *M. tb*, which necessitates the investigation of innovative approaches for disease treatment and prevention.

*M. tb* infection of macrophages

*M. tb* is a highly successful, facultative intracellular bacterium of mononuclear phagocytes. Establishment of a successful infection requires that *M. tb* bacilli enter macrophages through phagocytosis and survive within distinctive phagosomes. Intraphagosomal survival is determined by the ability of *M. tb* to circumvent the normal mechanisms of destruction for phagocytosed pathogens [239]. Survival strategies for *M. tb* include disruption of the inflammatory response, suppression of ROS and RNS, and arrest of phagosomal maturation and acidification [230]. Pathogen manipulation of host machinery can begin with the first interaction between *M. tb* and the macrophage at the plasma membrane where surface receptor activation can dictate infection outcome. *M. tb* cell wall components are evolved to differentially activate a subset of
macrophage surface and intracellular receptors. Those receptors include: TLRs, mannose receptor (MR), dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN), complement receptor (CR), scavenger receptors (SR), dendritic cell-associated C-type lectins (Dectin I/II), macrophage-inducible C-type lectin (Mincle) and nucleotide-binding oligomerization domain-like receptors (NOD1/2). Through these interactions *M.tb* influences the inflammatory balance and intracellular trafficking in order to promote bacterial survival [239]. During engulfment, *M.tb* achieves phagosomal arrest in part through MR-dependent phagocytosis that does not induce inflammation and targets its cargo to the early endosomal compartment. *M.tb* thereby limits phagosome-lysosome fusion and disrupts host protective cytokine production [240]. *M.tb* maintains the phagosome at a pH of 6.4 [241] and cultivates properties similar to the early endosome further preventing its maturation along the lysosomal pathway [242]. *M.tb* specifically reduces recruitment of early endosomal auto-antigen 1 (EEA1) [243] and thereby prevents Rab GTPase conversion from Rab5 to Rab7 on the phagosome, thereby preventing maturation [244, 245].

Cytokine signaling is among the most important factors governing *M.tb* eradication and as such *M.tb* has evolved to manipulate macrophage cytokine production in its favor. The host immune response can use cytokine signaling to clear infection by enhancing bacterial killing mechanisms such as ROS/RNI generation. TNFα is important for control of infection; it induces inflammation and
is critical to formation of the granuloma. Its loss in mice reduces host resistance and increases \textit{M. tb} dissemination [246]. In that context, TNF\(\alpha\) blocking therapies increase \textit{M. tb} susceptibility and reactivation [247]. IL-6 is another pro-inflammatory cytokine important for control of \textit{M. tb} infection in mice [248] but it along with IL-1\(\beta\) is also associated with disease progression [249]. Despite this phenomenon IL-1\(\beta\) is required to maintain resistance to \textit{M. tb} in mice [250]. It is required to induce iNOS [251] and is induced through C/EBP\(\beta\) in response to mycobacterial lipoarabinomannan (LAM) [252]. Polymorphisms in the C/EBP\(\beta\) binding site on the IL-1\(\beta\) promoter enhance its expression and lead to increased TB reactivation, severity and poor treatment outcome [253]. IL-10 polymorphisms are also associated with TB susceptibility. IL-10 is increased in the lavage fluid and serum of active TB patients and increased IL-10 levels in sputum positively correlate with \textit{M. tb} antigen load. Further, it reduces T cell proliferation and IFN-\(\gamma\) production and blocks phagosome maturation in MDMs and AMs [239].

Modulation of these and other cytokines during \textit{M. tb} infection has dramatic impact on macrophage host defense and \textit{M. tb} growth and survival. Therefore understanding the potential impact of zinc on their production during infection is essential to resolving the complexity of the host-pathogen interaction. Cytokine responses are often divergent between models. In particular dissimilar susceptibility and species-specific responses to \textit{M. tb} infection between human and murine macrophages [254] necessitates investigation of the infection in
primary human cell models which are more physiologically relevant and valuable to the understanding of human health.

**Micronutrients and Tuberculosis**

Prior to the introduction of antibiotic chemotherapy TB treatment primarily consisted of fresh air, sunlight and intermittent dosing with cod liver oil [255]. Cod liver oil is rich in vitamin A and vitamin D and the latter is converted to an active form by sunlight. Supplementation of either agent inhibits *M.tb* growth in macrophages [256, 257] underscoring the fact that micronutrient therapy was historically a major therapeutic modality for TB treatment. Additionally, active TB is associated with reduced circulating levels of both vitamin A [258] and D [259-261] and reduced levels of the antioxidant vitamins C and E [262]. Vitamin D deficiency is associated with TB disease prevalence and severity [259, 263]. In human macrophages activation by IFN-γ and subsequent production of antimicrobial cathelicidin and fusion of the *M.tb* containing phagosome with the lysosome is vitamin D-dependent [264]. Although antibiotic therapy remains the mainstay of TB treatment, the importance of micronutrient-dependent host defense against *M.tb* deserves further attention given their pleotropic and potentially positive impact on immune function.

Trace element redistribution is a critical host defense strategy against *M.tb*. As a result of systemic redistribution, TB is associated with anemia of chronic disease which results in macrophage iron loading [185]. Although
protective against extracellular pathogen growth, iron loading of macrophages may be beneficial to *M.tb*. Impaired access to intracellular labile iron reduces growth of *M.tb* in macrophages from patients with hereditary hemochromatosis [204] a disease which disrupts iron accumulation due to elevated ferroportin 1 export [265]. Previously described in detail was the host defense strategy of hyper-concentration of iron and copper into the phagosome through NRAMP1 and ATP7A in macrophages to limit mycobacterial growth [211, 266]. However, *M.tb* actively up-regulates mycobacterial copper transport protein B (MctB) which rescues it from copper toxicity [266]. Iron is also exported from the mycobacterial phagosome by ferroportin 1 [205] which can result in phagosomal iron deprivation during IFN-γ activation [214]. Iron is essential for *M.tb* and is collected into the *M.tb* phagosome from intracellular and extracellular stores [203, 267]. It can be captured from transferrin or lactoferrin by mycobacterial siderophores including carboxymycobactins and exochelin or by heme import [268]. In addition to iron and copper, zinc redistribution strategies are also employed by macrophages as a host defense against *M.tb*. Zinc status and metabolism may have critical importance to macrophage effector functions during *M.tb* infection.
Zinc & *Mycobacterium tuberculosis*

The vast majority of TB infections occur in impoverished nations, where zinc deficiency is commonplace. Zinc is an essential micronutrient, deficient in the diets of many indigent populations in TB endemic areas due to reduced access to foods rich in absorbable zinc [93]. Zinc deficiency is a major cause of immune dysfunction and infection, affecting nearly one-third of the world’s population [93] and has a similar global distribution to TB. Studies investigating zinc supplementation strategies during active TB have revealed no convincing evidence to substantiate the benefit of zinc supplementation in TB patients [269]. Many of these studies have inadequate samples sizes, determined zinc status and TB using potentially inaccurate methodologies and were complicated by comorbid conditions including PEM and HIV. However one small study did observe increases in TNFα and IFN-γ and reduction of IL-10 mRNA expression in whole blood from zinc supplemented TB patients that was associated with sputum smear conversion [270]. *M.tb* infection does indeed influence human zinc metabolism as serum zinc concentrations are significantly reduced in patients with active TB disease [271]. Furthermore, animal models of zinc deficiency demonstrate reduced T cell immunity in response to *M.tb* infection [272]. Cumulatively, these findings indicate an epidemiological link between TB incidence and the risk for zinc deficiency.

At a cellular level, cytosolic zinc concentrations have been shown to increase in human macrophages following infection with *M.tb* [55] and are
associated with hyper-concentration of zinc in the *M.tb* phagosome [214]. These alterations lead to up-regulation of the mycobacterial zinc efflux pump CtpC, which is required for *M.tb* survival at high zinc conditions. Furthermore increased expression of MT and ZnT1 through MTF-1 indicate that *M.tb* infection induces cytosolic zinc influx in macrophages [55]. ZIP8 is induced by mycobacterial infection in human monocytes. It functions to elevate cytosolic zinc concentrations and localizes to the liposomal pathway in response to mycobacterial cell wall extracts [66]. Given the significant impact of ZIP8 on innate and adaptive immune function [30, 33], we propose that evaluation of ZIP8 relative to *M.tb*-induced alteration of macrophage function is warranted.
1.5. Goals for the dissertation

Central Hypothesis and Specific Aims

The ultimate goal of the research presented herein is to identify the role of macrophage zinc metabolism and in particular ZIP8 in TB pathogenesis. Improved understanding of this important biology has the potential to eventually generate new ideas for therapy and thus contribute to the eradication of TB along with its associated morbidity and mortality. We propose that ZIP8, its expression, cellular distribution and ZIP8-dependent zinc flux in *M. tb* infected macrophages plays a central role in determining the outcome of infection. Based upon the extensive body of scientific literature reviewed above, we formulated the central hypothesis that zinc metabolism modulates macrophage host defense through ZIP8 during infection with *Mycobacterium tuberculosis*. This hypothesis was rigorously examined by pursuing the following three aims. The first aim, which will be explored in chapter two, established a physiologically relevant zinc optimum for *in vitro* modeling of ZIP8-dependent human macrophage host defense and evaluated the relationship between zinc, ZIP8 and NF-κB. The second aim, discussed in chapter three, identified the impact of ZIP8-dependent zinc on human macrophage inflammatory signaling. The third and final aim, covered in chapter four, begins to identify the impact of zinc and ZIP8 on macrophage inflammatory host defense functions and *M.tb* growth during infection.
Figure 1. Human zinc transporters The general zinc trafficking pattern and intracellular distribution of ZIP and ZnT transporters.
CHAPTER 2: THE RELATIONSHIP BETWEEN ZIP8, MACROPHAGE ZINC STATUS, AND NF-κB SIGNALING

2.1 Summary

Before investigating the impact of ZIP8 on macrophages during *M.tb* infection we sought to develop a durable model system of human macrophage zinc metabolism that is responsive to pathogen stimulation. Human zinc metabolism, which is primarily controlled by fourteen ZIP cytosolic zinc import proteins and ten ZnT cytosolic zinc export proteins, is altered by microbe-initiated activation of innate immune cells [42, 217]. LPS derived from the outer membrane of gram-negative bacteria is a potent immune activator of macrophages. LPS stimulates human macrophage gene transcription following Toll-like receptor 4 (TLR4) binding and sequential activation of intracellular biochemical signaling cascades. The resulting nuclear localization and activation of a number of transcriptional co-activators and transcription factors including NF-κB, largely determines the inflammatory response to infection [178]. In a previous publication our group discovered that ZIP8 is induced through the canonical NF-κB pathway following LPS exposure. Additionally, we found that ZIP8, which is constitutively expressed and highly inducible in macrophages
[181], is located on macrophage plasma membranes and intracellular vesicles [33]. Human monocytes do not abundantly express ZIP8 [66]; however, in THP1 cells, a human monocyte cell line, the low level of constitutive ZIP8 that does exist is important for zinc import prior to the induced pool of ZIP8 [33]. Furthermore, zinc import by ZIP8 is responsible for reduction of LPS induced NF-κB transcriptional activation through inhibition of I kappa-B kinase (IKK) activity, thereby tempering monocyte inflammation [33, 180]. Based upon our previous work and the work of others, we hypothesized that macrophage zinc metabolism is inflammation- and zinc status-dependent and impacts NF-κB activity in part through ZIP8.

Through the course of the investigation we determined that LPS exposure alters macrophage ZIP8 expression and cytosolic zinc accumulation and also that zinc influences NF-κB activity. Using a small molecule inhibitor and overexpression systems, we observed that ZIP8 is inducible through NF-κB and ZIP8-dependent zinc is capable of NF-κB inhibition in cell lines. Then by shifting our focus to a more physiologically relevant MDM model of primary human macrophages, we determined that zinc transporter expression, particularly that of ZIP8 is responsive to LPS in macrophages. Given that ZIP8 functions primarily as a cytosolic importer of zinc [69], we then evaluated the impact of LPS on macrophage intracellular zinc accumulation by comparing the influence of LPS and zinc supplementation on intracellular zinc accumulation in macrophages that had been cultured in zinc depleted media or control media over a prolonged
period (7 days). Subsequently we determined that macrophage zinc status modulates zinc import such that cellular zinc deprivation increases LPS-induced macrophage zinc uptake potential. Knowing this, we then evaluated the impact of ZIP8 on LPS-induced macrophage zinc accumulation. Using a new siRNA-mediated ZIP8 knockdown model in MDMs, we determined that macrophage zinc accumulation during zinc supplementation is independent of LPS exposure and ZIP8. Furthermore, we showed that zinc supplementation of human macrophages within the physiological range induces NF-κB activity, an effect that is independent of ZIP8. However, we unexpectedly determined that ZIP8 does inhibit NF-κB in the absence of zinc supplementation, indicating that ZIP8 may play a role in redistribution of intracellular zinc pools. Accordingly, below we describe a new model of inflammation-dependent macrophage zinc responsiveness that is distinct from our previously described findings in human monocytes and monocytic cell lines [33].
2.2 Results

The relationship between ZIP8 and NF-κB in cell lines

In preliminary experiments we used human cell lines including the lung epithelial cell line A549, the embryonic kidney cell line HEK293 and the monocytic cell line THP1 to recapitulate previous reports [33, 66, 68] of LPS-dependent ZIP8 induction through NF-κB and subsequent ZIP8-specific modulation of NF-κB activity. When treated with the IKK inhibitor Bay 11-7082 and exposed to LPS, THP1 cells fail to express ZIP8 mRNA and the NF-κB dependent cytokine IL-1β. ZnT1 expression, which is induced by cytosolic zinc influx is also increased by LPS and reduced following NF-κB pathway inhibition (Fig 2A). ZIP8 and IL-1β expression is highly inducible by TNFα in A549 cells and by LPS in THP1 cells. In contrast, expression of ZIP4 another plasma membrane-bound zinc importer, which is zinc responsive, is insensitive to inflammation (Fig 2B). Based upon this observation we sought to evaluate the ZIP8 specificity of ZIP-dependent NF-κB inhibition. To do so, ZIP8 or ZIP4 were over-expressed in HEK 293 cells (Fig 2C), which demonstrated that both transporters inhibit NF-κB activity after 8 h of TNFα exposure with and without zinc supplementation (Fig 2D). Despite the abundance of both ZIPs, neither significantly increased intracellular zinc accumulation after 24 h (Fig 2E).
ZIP8 is constitutively expressed and uniquely induced by LPS in human macrophages

Import of zinc into the cytosol is achieved mainly through fourteen ZIP transporters which are differentially regulated and expressed in distinct cell types as a function of intracellular and extracellular cues [42]. In particular, ZIP8 is typically not highly expressed constitutively but is inducible by inflammation in monocytes [66]. In order to evaluate the potential impact of ZIP8 on host defense in a relevant model of human tissue macrophages, we first examined mRNA and protein ZIP8 expression during the process of primary human monocyte to macrophage differentiation. During differentiation ZIP8 mRNA (Fig 3A) and protein (Fig 3B) are constitutively expressed. Consistent with previously published reports in lung epithelial cell lines [68], ZIP8 appeared as a heavily glycosylated, protein with an apparent molecular mass of approximately 140 kDa as well as a 55 kDa cytosolic isoform as determined by Western blotting. Those bands disappeared when competed with the peptide antigen used for antiserum generation (Fig 3C). Upon evaluation of the 140 kDa band by protein mass spectrometry it was revealed that potential protein binding partners exist for ZIP8 including a membrane bound phosphatase, receptor-type tyrosine-protein phosphatase C, the iron binding ferritin light chain protein as well as the siderophore sequestration protein lipocalin-1 (data not shown). At this point, we chose not to pursue the potential for protein binding partners. Next, we determined the capacity of LPS to influence the mRNA expression of all ZIPS (1-
in MDMs from individual donors by qRT-PCR. We observed that LPS induces macrophage ZIP8 expression (Fig 3D) without significantly altering mRNA expression of the thirteen other ZIPs in MDMs (Fig 3E). ZIP8 was constitutively expressed in resting MDMs (Fig 3F) and ZIP8 mRNA was significantly induced following LPS exposure by over 10-fold, which led to increased ZIP8 protein production in MDMs over time (Fig 3D and 3F). Following LPS exposure, macrophage ZIP8 mRNA levels increased by 6 h and peaked at 48 h while protein levels increased at 24 h and remained elevated through 48 h (Fig 3D and 3F). During the course of this investigation our group published that LPS in MDMs induces ZIP8, where it localizes to intracellular vesicles and the plasma membrane [33].

Establishment of a model of macrophage zinc deficiency

Armed with the knowledge that macrophage zinc metabolism is inflammation responsive [33] and that zinc deficiency disrupts the coordination of the inflammatory response to infection [75], we next developed an in vitro model of macrophage zinc deficiency to aid in the exploration of zinc status on host defense at a cellular level. We began by determining the impact of various modifications to culture media on zinc content by atomic absorption spectroscopy (AAS). RPMI cell culture media alone, which has no zinc added [273] contained only 1 µg/dL more zinc than double distilled water and surprisingly only 1 µg/dL less than RPMI supplemented with 2% or 10% human serum. In RPMI with 10%
human serum, zinc depletion using the specific insoluble chelator Chelex-100 completely eliminated measureable zinc. High dose zinc supplementation into RPMI with 10% human serum resulted in supra-physiological zinc levels in the media (Fig 4A). We next determined that ZIP8 induction is independent of extracellular zinc. Macrophage zinc deficiency achieved by prolonged culture of MDMs for 7 days in zinc-depleted media did not alter LPS-induced ZIP8 protein expression as determined by Western analysis after 24 h (Fig 4B). Upon evaluation of macrophages cultured in RPMI with 10% autologous serum by confocal fluorescence microscopy, 6 h LPS exposure increased intracellular zinc levels as determined with the zinc specific fluophore Zinpyr-1. That effect is eliminated in zinc-depleted conditions. Further when supplemented with zinc to physiological levels, zinc accumulation in zinc-deficient LPS-exposed macrophages increases dramatically (Fig 4C). Finally, we observed that cellular zinc deficiency does not alter the integrity of macrophage monolayer cell numbers as determined by nuclei enumeration (Fig 4D), cell viability as determined by trypan exclusion (Fig 4E) or cytotoxicity as determined by lactate dehydrogenase release (Fig 4F). Collectively, these results confirm our capacity to model physiological insufficient and sufficient zinc conditions in macrophages.
ZIP8 does not alter in vitro macrophage zinc accumulation

In order to evaluate the impact of ZIP8 on macrophage zinc accumulation we reduced ZIP8 production in MDMs by transfection of anti-ZIP8 siRNA. This leads to a reliable and reproducible knockdown of ZIP8 without compromising monolayer integrity as determined by nuclei enumeration (Fig 5A). In this model, ZIP8 mRNA is reduced by 400% (Fig 5B) consistently at 24 h following LPS stimulation as quantified by qRT-PCR. ZIP8 protein knockdown in MDMs was verified by Western blot showing a consistent and durable knockdown of greater than 80% below control levels at 6 h and 60% at 48 h (Fig 5C) following LPS stimulation. Importantly, other than a reduction in ZnT1 at 24 h, expression of all other zinc transporters is not significantly altered as a consequence of ZIP8 knockdown (Table 1) during LPS exposure. Consequently, compensatory changes in the expression of other ZIPs do not significantly contribute to the findings in these or subsequent studies. We next evaluated the impact of ZIP8 on macrophage zinc content using the ZIP8 knockdown model. Supplementation of zinc at the upper end of the physiological range (18 µM) for 6 h increases extracellular zinc concentrations (Fig 5D) as determined by AAS and also increases intracellular zinc levels (Fig 5D, 5E and 5F) shown by AAS and flow cytometry by detecting Zinpyr-1 fluorescence. However, LPS exposure and/or the presence of ZIP8 do not substantially impact intracellular zinc accumulation under the conditions studied (Fig 5D, 5E and 5F).
**Zinc and ZIP8 individually regulate macrophage NF-κB activity**

Next, we determined whether ZIP8-dependent zinc influences macrophage NF-κB activity. We discovered that at early time points (within 1 h) of LPS activation, LPS and zinc increase the binding activity of both p50 and p65 subunits of NF-κB and with a synergistic effect (Fig 6A) as determined by TransAM ELISA. Additionally, total protein content of the NF-κB binding protein p105 and its degradation product p50 or p65 were unaltered by zinc supplementation during LPS activation between 5 min and 1 h (Fig 6B) as determined by Western blot. Next, using the previously established knockdown model (Fig 5A, 5B and 5C), we observed that ZIP8 reduces p50 and p65 activity initially at 30 min following LPS activation in the absence of zinc but not thereafter out to 2 h as determined by TransAM ELISA (Fig 6C). Furthermore, total cellular content of NF-κB binding proteins p105 and IκBα are not altered substantially during zinc supplementation of LPS-exposed macrophages when compared to LPS stimulation alone out to 2 h. However in the absence of zinc supplementation, ZIP8 knockdown increases total levels of NF-κB subunits p50 and p65 at 30 min after LPS as determined by Western blot (Fig 6D). These results revealed zinc-dependent pro-inflammatory activation of NF-κB that is independent of ZIP8. In comparison ZIP8 has an opposing inhibitory effect on NF-κB that is eliminated by increased extracellular zinc concentrations.
2.3 Discussion

In a previous publication we showed that constitutively present ZIP8 is responsible for early zinc influx and IKKβ-dependent NF-κB inhibition in LPS-exposed THP1 cells. We also determined that ZIP8 is induced through NF-κB-dependent transcription in response to LPS stimulation in those cells [33]. Here we sought to determine whether macrophage zinc metabolism is influenced by inflammatory stimulation. Our primary focus was to determine the impact of ZIP8 and zinc on macrophage zinc status and NF-κB activity in primary human macrophages. Consistent with our previous published work [33], ZIP8 is induced through NF-κB in THP1 cells in response to LPS (Fig 2A and 2B). Further the LPS-induced response is recapitulated in human macrophages (Fig 3D, 3E and 3F) indicating that NF-κB potentially also drives macrophage ZIP8 expression. ZIP8 has the highest baseline expression among the 14 ZIP transporters in MDMs and is uniquely responsive to LPS (Fig 3E). These findings identified ZIP8 as the predominant LPS-inducible macrophage zinc importer. In the initial report detailing the discovery of ZIP8 (originally identified as BIGM103), the authors reported baseline ZIP8 mRNA expression in human DCs and M-CSF- or GM-CSF-elicited macrophages but no mRNA expression and low resting protein expression of ZIP8 in monocytes [66]. Consistent with this report we found that MDMs produce appreciable amounts of ZIP8 protein at rest (Fig 3F), indicating that ZIP8 has the capacity to modulate intracellular zinc levels both within resting macrophages and to a greater extent following LPS exposure. Furthermore ZIP8
mRNA and protein expression is elevated throughout the process of monocyte to macrophage differentiation (Fig 3A and 3B). We propose that the constitutive presence of ZIP8 is due in part to changes in the phenotype of mononuclear phagocytes that occurs during differentiation to macrophages [274]. ZIP8 up-regulation in human macrophages may have critical importance to host defense. Similar to monocytes, ZIP8 up-regulation in macrophages may contribute to intracellular zinc redistribution during systemic infection that generates a vascular zinc deficiency. That hypozincemia is hypothesized to reduce zinc availability to extracellular pathogens [75]. Accordingly, ZIP8-dependent zinc uptake by tissue macrophages may act as a mechanism to deprive extracellular pathogens of zinc in the local tissue microenvironment. Further, ZIP8 localization to intracellular vesicles and the plasma membrane [33] may also facilitate intracellular zinc redistribution away from intracellular pathogens as a mechanism of host defense [218].

In comparison to ZIP8 we found that expression of the zinc exporter ZnT1 is also induced in response to LPS in THP1 cells (Fig 2A) and primary human macrophages (Table 1). This effect may be due in part to ZnT1 induction through MTF-1 in response to increases in intracellular zinc [52, 57], which indicates that LPS induces early zinc waves through another constitutively expressed zinc transporter. ZIP8-dependent zinc activates MTF-1 transcription in murine chondrocytes [72]. Furthermore, our group has shown previously that ZIP8 is responsible for early zinc waves between 30 min and 2 h in LPS-exposed THP1
cells [33]. Here we determined that ZIP8 knockdown increases ZnT1 expression in response to LPS (Table 1) but not before 24 h. Taken together, this suggests that additional mechanisms may account for zinc import and contribute to this effect.

Although we found that ZIP8 is capable of inhibiting NF-κB in cell lines following over-expression (Fig 2C), this effect is not specific to ZIP8 as ZIP4 over-expression also inhibits NF-κB but to a greater extent (Fig 2D). ZIP4 localizes to the plasma membrane similarly to ZIP8 [44] however its expression is not responsive to inflammatory signals (Fig 2B). The relatively greater effectiveness of ZIP4 in suppressing NF-κB compared to ZIP8 in our over-expression model is likely due to its higher affinity for zinc [44, 275]. The ability of ZIP transporters to impact NF-κB signaling is not exclusive to ZIP8 and the relative abundance of constitutive and inducible ZIP proteins may be the key determining factor. Surprisingly, overexpression driven by viral promoters of ZIP8 and ZIP4 in HEK cells (Fig 2C) did not lead to increases in intracellular zinc concentrations after 24 h of zinc supplementation as determined by fluorescence quantification of the zinc-specific fluorophore FluoZin-3 [276] (Fig 2E). The apparent lack of change at that later time point may be due to unknown compensatory alterations in zinc metabolism.

Zinc and serum content of cell culture media can have a substantial impact on mononuclear cell zinc uptake potential and subsequent zinc-dependent responses [277, 278]. Our analysis of the culture media used in the
MDM model verified that RPMI, which has no zinc deliberately added during formulation [273], has minimal zinc contamination that is similar to the zinc content of distilled water (Fig 4A). Further, the addition of 2 or 10% autologous human serum to RPMI only marginally increased the total zinc content of the media whereas high dose zinc supplementation elevated the culture media content above the physiological range of 70-120 µg/dL (Fig 4A). The lack of an observable difference in zinc concentration with the addition of serum is likely due to the limitations of AAS. The zinc binding capacity among different serum sources and concentrations can potentially be markedly different based largely on differences in albumin [277]. Further, in our hands, the zinc pool within the culture media was completely chelatable (Fig 4A). We leveraged our ability to deplete culture media of zinc content to design a system of macrophage cellular zinc deficiency following a 7 day culture of MDMs in zinc-chelated media [279]. Using the zinc-specific fluorophore Zinpyr-1 [280], we found that LPS induces intracellular zinc accumulation in macrophages after 6 h of exposure, particularly in zinc-deficient macrophages that are cultured in zinc-supplemented media (Fig 4C). LPS-induced zinc uptake by zinc-deficient macrophages supplemented with exogenous zinc produces a diffuse staining pattern with punctate inclusions that are characteristic of zinc supplementation in other cell types [281]. Macrophage cellular zinc deficiency did not impact the number, viability or cytotoxicity of macrophages (Fig 4D, 4E and 4F), indicating that this model allows for exploration of zinc deficiency on macrophage host defense properties. However,
depletion of zinc in media using Chelex-100 has been shown to augment cytokine production in monocytes [150] and therefore may not be an ideal system for the investigation of macrophage inflammation. Because of the relatively low levels of zinc within our standard MDM culture conditions (Fig 4A), this experimental system may represent an alternative and suitable model to study the impact cellular zinc deficiency.

Creation of a ZIP8 knockdown model in MDMs provided the capacity to determine the impact of ZIP8 on intracellular zinc content. Although the siRNA nucleofection method of electroporation does reduce macrophage numbers to some extent, this reduction is equivalent to the scramble control group through 72 h (Fig 5A). Furthermore, a knockdown efficiency of over 60% is sustained in macrophages following LPS exposure (Fig 5B and 5C). Knockdown of ZIP8 during LPS exposure minimally impacted the expression of other dedicated zinc transporters, with the exception of ZnT1 (Table 1). Cellular compensation in ZIP expression in response to reduction of ZIP8, which could confound results, did not occur. Extracellular (Fig 5D) and intracellular (Fig 5D and 5E) zinc levels were increased by supplementation irrespective of ZIP8 knockdown or LPS-exposure. This observation indicates that zinc entry into macrophages following LPS exposure is largely independent of ZIP8. Due to the fact that knockdown was incomplete (Fig 5C), the residual pool of ZIP8 may be enough to prevent an observation of a ZIP8-dependent effect. Further, analysis of intracellular zinc accumulation beginning at 6 h following zinc supplementation may have missed
the initial window for the first LPS-induced ZIP8-dependent zinc wave. Previous reports have demonstrated zinc mobilization within 20 min following stimulation, which may represent an initial re-equilibration of macrophage zinc status [33, 151]. Furthermore, zinc associated with cell signaling constitutes no more than 10% of the entire intracellular pool, such that analysis of intracellular alterations may be beyond the capability of AAS to discriminate [32]. In contrast, using an alternative approach, Zinpyr-1 fluorescence by flow cytometry did indicate a trend toward reduced uptake of zinc during supplementation in ZIP8 knockdown (Fig 5F).

During LPS exposure, zinc supplementation in the upper normal physiologic range enhances early NF-κB signaling in human macrophages similar to monocytes and cell lines [151]. Zinc increases p50 and p65 activity in the nucleus (Fig 6A) but does not alter degradation of the p50 precursor p105 or total levels of p50 or p65 (Fig 6B) within 1 h after LPS. Those results suggest that the additional activity of NF-κB does not result from increased degradation of inhibitory binding proteins or production of NF-κB subunits. The early pro-inflammatory zinc effect may be due in part to increased tonicity as a result of elevated salt concentrations due to supplementation [282] or zinc-dependent increases in LPS binding efficiency [283]. Moreover, these findings do confirm that macrophages have the capacity to use environmental zinc to enhance pro-inflammatory signaling [152].
Similar to our previously reported results in human monocytes and cell lines, ZIP8 reduces NF-κB in human macrophages at 30 min following LPS stimulation. ZIP8 knockdown increases p50 and p65 activity in the nucleus (Fig 6C) as well as their total cellular content (Fig 6D) during LPS exposure. ZIP8-dependent reduction in NF-κB is abrogated by zinc supplementation (Fig 5C). In fact, zinc supplementation increases p50 and p65 nuclear activity regardless of the presence of ZIP8 (Fig 6A and 6D). These findings reveal a multifaceted impact of zinc metabolism on macrophage NF-κB activity. Import of extracellular zinc activates NF-κB transcription independently of ZIP8, presumably through a different import mechanism. Conversely, ZIP8 which is induced through NF-κB, subsequently reduces NF-κB in a negative feedback loop, an effect that is negated by opposing NF-κB activation during zinc supplementation. Thus, regulation of zinc metabolism in macrophages and its impact on innate immune function are more complicated than we originally predicted. This work underscores the complexity of this dynamic, phagocytic cell that resides at the forefront of the host-pathogen interface.
2.4 Materials and methods

Reagents
TRIzol, RPMI 1640 with L-glutamine, DPBS, Trypan blue and FluoZin-3 AM were purchased from Invitrogen (Carlsbad, CA). Zinc sulfate heptahydrate, recombinant TNFα, LPS L-439 and L0923, Napthol blue, Zinpyr-1, Bay11-7082 and BSA were purchased from Sigma-Aldrich (St. Louis, MO). Ficoll-Paque and TMB substrate were purchased from GE Healthcare (Little Chalfont, UK). Rabbit polyclonal antiserum anti-peptide to amino acid residues 225-243 of human ZIP8 (1:1000) was purchased from Covance (Princeton, NJ). Mouse anti-human monoclonal β-Actin (#69101) (1:10,000) antibody was purchased from MP Biomedicals (Santa Ana, CA). Rabbit anti-human polyclonal NF-κB p65 (#372) (1:1000) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-human monoclonal NF-κB1 p105/p50 (#12540) (1:2000) and IκBα (#32518) (1:1000) were purchased from Cell Signaling Technology (Danvers, MA). ZIP8 epitope specific, 21mer small interfering RNA (siRNA) (target sequence TAGGACTTAGGAAATAAATAA) and scramble control siRNA were purchased from QIAGEN (Hilden, DEU). Chelex-100 resin was purchased from BioRad (Berkeley, CA).

Cell line culture
A549, HEK293 and THP-1 cells were routinely maintained in standard culture conditions in RPMI-1640 with 10% FBS at 37°C in 5% CO2. Cells were washed
once before exposure to Bay 11-7082 (10µM) 30 minutes prior to LPS (100 ng/mL) or TNFα (10 ng/mL).

**Plasmid transfection and NF-κB Luciferase assay**

An SV40 driven SLC39A4 cDNA plasmid and empty vector generously provided by the laboratory of Glen Andrews, SLC39A8 cDNA plasmid pCMV6-XL4_SLC39A8 and empty vector (Origene) or a luciferase construct containing 3 κB sites was utilized as a reporter for NF-κB activation were transfected into cells using Lipofectamine 2000 (Invitrogen) per manufacturer’s instructions. The luciferase assay was performed per the manufacturer’s instructions using the Dual-Glo system (Promega).

**Human monocyte-derived macrophages**

All human subject research was conducted using an IRB approved by the Ohio State University’s Office of Responsible Research Practices and with written donor consent. Human monocyte-derived macrophages (MDMs) were isolated and differentiated from PBMCs as previously described [284]. Briefly, after collection of blood from healthy human volunteers PBMCs were isolated from heparinized blood using Ficolle-Paque (GE Healthcare) density gradient centrifugation and buffy-coat extraction into Teflon wells (Savillex) followed by a 1 to 5 day differentiation period following incubation in RPMI-1640 culture medium (RPMI) (GIBCO) with 20% autologous serum at 37°C in 5% CO₂. Wells
were then placed on ice for 30 min and PBMCs were removed by washing with RPMI. MDMs (4x10⁵/mL) were adhered in 6- to 24-well tissue culture plates (Falcon) for 2-3 h at 37°C in 5% CO₂ in RPMI-1640 with 10% autologous serum. Monolayers were then washed to remove lymphocytes and supplemented with RPMI-1640 with 10 - 20% autologous serum and incubated 1 to 7 days.

**Zinc deficiency, supplementation and LPS exposure**

Autologous serum was incubated overnight at 4°C with rocking in Chelex-100 resin (BioRad). MDM monolayers were cultured in RPMI with 20% zinc normal or zinc-depleted autologous serum and cultured 7 days. Macrophages were washed and repleted with RPMI containing 2% autologous serum, which contains < 1 µM zinc, then repleted with or without 18 µM zinc sulfate, with or without LPS 100 ng/mL.

**Cytotoxicity assay**

Lactate dehydrogenase activity assay (SigmaAldrich) was conducted according to the manufacturer’s instructions.

**Protein lysate preparation and Western blot**

MDM monolayers were lysed with TN1 buffer to generate whole cell lysate, then incubated at 4°C for 10 min. Lysates were centrifuged at 17,949 × g at 4°C to remove cell debris. To generate nuclear isolates, MDM monolayers were lysed
and processed with NE-PER nuclear and cytoplasmic extraction reagents (ThermoFisher Scientific) per manufacturer’s instructions. Protein concentration was measured using the Pierce BCA-protein assay kit (ThermoFisher Scientific) per manufacturer’s instructions. Lysates were reduced, denatured and separated by SDS-PAGE, then transferred onto nitrocellulose membranes and blocked with 5% milk in TBS-T. Those membranes were then probed with primary and secondary antibodies of interest and by development using ECL (GE Healthcare). Band densitometry was measured using Image J software. Intensity was determined by subtracting background intensity compared to β-actin or Lamin B1.

**Quantitative Real Time RT-PCR**

MDM monolayers were lysed with TRIzol (Invitrogen). RNA was isolated using chloroform extraction and ethanol precipitation then converted into cDNA using the ThermoScript RT-PCR system (Invitrogen). Quantitative PCR was performed using SYBR Green (Applied Biosystems). Genes of interest were normalized to GAPDH. Relative copy number (RCN) was determined using the formula: 

\[ \text{RCN} = 2^{-\Delta \text{Ct}} \times 100 \]

where \( \Delta \text{Ct} \) is the \( \text{Ct}_{\text{target}} - \text{Ct}_{\text{reference}} \). Fold change was calculated by comparing treatment groups to resting controls.
Transfection of MDMs

Following differentiation, PBMCs containing MDMs were transfected with 50 nM ZIP8 or control siRNA using the Amaza Nulceofector (Lonza) [285] as directed by the manufacturer. Following transfection PBMCs were seeded onto tissue culture plates and incubated in RPMI-1640 with 10% autologous serum for 2 h at 37°C in 5% CO₂. Lymphocytes were removed by washing with warm RPMI-1640. Adhered, transfected MDMs were repleted with RPMI-1640 containing 20% autologous serum and incubated overnight at 37°C in 5% CO₂ to allow recovery before further treatment.

Confocal microscopy

Cells were cultured on glass coverslips, then following treatment were exposed to FluoZin-3 AM (1µM) or Zinpyr-1 (1µM) for 30 min then washed three times with DPBS and fixed to slides using Prolong Gold (ThermoFisher). The slides were then examined by confocal microscopy (Olympus BX61).

Atomic absorption

Samples were dissolved with nitric acid (1%) for 24 hours then diluted in MilliQ water and assayed by atomic absorption spectroscopy using AAnalyst 400 (PerkinElmer).
**Flow cytometry**

After treatment macrophages were washed w/ RPMI then repleted with RPMI with 5µM Zinpyr-1 and incubated for 30 min, then washed 3 times with DPBS and placed on ice for 30 min. Cells were then gently removed with a rubber policeman into a microcentrifuge tube on ice. Flow cytometry analysis was performed using FACSConto II (BD) and analyzed with Flow Jo (Treestar).

**Activity Assay**

Nuclear lysates were obtained from MDMs following treatment or control conditions as previously described using NE-PER nuclear and cytoplasmic extraction reagents purchased from Thermo Scientific (Rockford, IL) per manufacturer's instructions. Briefly, cells were lysed with ice cold cytoplasmic extraction reagent I for 10 min at 4°C with rocking then vortexed for 15 sec and placed on ice for 10 min. Next cytoplasmic extraction reagent II was added and samples were vortexed for 5 sec, then placed on ice for 1 min followed by vortexing for 5 sec and centrifugation at 16,000xg for 5 min at 4°C. Ice cold nuclear extraction reagent was added to the insoluble pellet and vortexed for 15 sec followed by placement on ice for 10 min, repeated 4 times followed by centrifugation at 16,000xg for 10 min at 4°C. Supernatants containing the nuclear extracts were then evacuated into fresh microcentrifuge tubes and stored overnight at -80°C. Nuclear localization of activated p65 and p50 in ice-thawed MDM nuclear lysates was then determined using the TransAM™ NF-κB
Transcription Factor Assay purchased from Active Motif (Carlsbad, CA) per manufacturer's instructions. Briefly, protein concentrations in nuclear lysates were determined by the Bradford assay using protein dye reagent concentrate purchased from Bio Rad (Hercules, CA). Recombinant protein standards or nuclear protein (2 µg/sample) in lysis buffer were loaded into wells on p50 or p65 oligonucleotide-coated plates containing binding buffer, then incubated at room temperature for 1 h with rocking. Following three washes wells were incubated with rabbit anti-human phospho-p50 or -p65 antibody (1:1000) in binding buffer for 1 h at room temperature. After three washes wells were incubated with anti-rabbit HRP-conjugated antibody (1:1000) in binding buffer for 1 h at room temperature. Wells were then washed 4 times and incubated with developing solution in the dark for 2.5 min before termination of the reaction with stop solution. Absorbance at 450 nm was then read by spectrophotometry.

Statistics

Each experiment was conducted a minimum of 3 times with different donors. Prism-5 software (Version 5.04; GraphPad) was used to determine the statistical significance of differences in means using one-sided ANOVA with Tukey’s post-test. \( p \) values < 0.05 were considered significant.
Figure 2. ZIP8 is induced by NF-κB and inhibits its function in cell lines.

(A) ZIP8, ZnT1 and IL-1β mRNA induction by two different formulations of LPS is inhibited by the NF-κB pathway inhibitor Bay11-7082 (10 μM) in THP1 cells after 4 h as determined by qRT-PCR. (B) ZIP8 but not ZIP4 expression is induced by TNFα (10 ng/mL) in A549 cells or LPS (100 ng/mL) in THP1 cells after 8 h as determined by qRT-PCR. Transfection of HEK293 cells with over-expression plasmids for ZIP8 or ZIP4 leads to an increase in protein production compared to vector controls as determined by Western blot (C). Overexpression results in a zinc supplementation-independent, zinc transporter-dependent decrease in TNFα (10 ng/mL)-induced NF-κB activity as determined by luciferase assay (D) but not an increase in intracellular zinc accumulation as determined by confocal fluorescence microscopy using FluoZin-3 (E). Panels A, B, C, D and E are single experiments conducted in triplicate (mean).
Figure 3. ZIP8 is constitutively present and highly inducible by LPS in human macrophages. ZIP8 mRNA (A) and protein (B) levels are maintained through monocyte to macrophage differentiation as determined by qRT-PCR and Western blot analysis. Cumulative densitometric analysis reveals that ZIP8 protein is constitutively present during differentiation. (C) The anti-ZIP8 antiserum is specific for bands at 55 and 140 kDa as determined by co-incubation with excess peptide used in the generation antiserum and analysis by Western blot. (D) ZIP8 mRNA in MDMs is significantly induced following LPS (100 ng/mL) exposure. (E) mRNA expression profile of all ZIPs reveals that LPS (100 ng/mL) exposure significantly increases expression of ZIP8 in MDMs over time as determined by qRT-PCR. In contrast, basal levels of the 13 other ZIPs were minimal and LPS exposure did not alter their expression. (F) ZIP8 protein levels in MDMs are significantly induced by LPS (100 ng/mL) exposure compared to resting unstimulated cells. Cumulative densitometric analysis reveals that ZIP8 protein levels increase two-fold by 24 h following LPS exposure. (A, D and E were quantified relative to GAPDH). Panels A, D and E are cumulative data from three different donors (mean ± SEM; ***p < 0.001). Panels B and F are each representative of 3 experiments. Panel C is a single experiment.
Figure 3

A

B

C

D

E

F
Figure 4. Prolonged culture in zinc deficient media results in macrophage zinc deficiency. (A) Zinc levels are marginal in RPMI with and without serum. Zinc is removed by chelation and increased by ZnSO$_4$ (100 µM) supplementation as measured by AAS. Prolonged culture (7 d) in zinc-depleted media does not impact TNFα (10 ng/mL)-induced ZIP8 protein levels after 24 h by Western blot (B) but does reduce intracellular macrophage zinc, and prevents cellular accumulation contributed by media in response to 6h LPS (100 ng/mL) stimulation; however macrophage zinc content is increased with ZnSO4 (10 µM) supplementation and LPS as determined by fluorescent confocal microscopy (C). Prolonged culture in zinc deficient conditions does not alter macrophage numbers (D), viability (E) or cytotoxicity (F) by napthol blue-black nuclei enumeration, trypan blue exclusion and LDH release assay, respectively. Panels A, B and C are single experiments. Panels D, E and F are each representative of 3 experiments conducted in triplicate (mean ± SD).
Figure 4

A

B

C

D

E

F

Figure 4
Figure 5. Constitutively expressed macrophage ZIP8 does not have a prolonged influence on intracellular zinc content. Macrophage numbers (A) are not altered in MDMs transfected with siRNA targeting ZIP8 compared to scramble control siRNA after 72 h. ZIP8 mRNA (B) and protein (C) levels are reduced following LPS (100 ng/mL) exposure and transfection with anti-ZIP8 siRNA vs. control. Densitometric analysis of Panel 4C reveals that ZIP8 protein levels are reduced by over 80% initially and then 60% at 48 h following knockdown. Both extracellular and intracellular macrophage zinc levels are unaffected after 6 h by ZIP8 knockdown or LPS (100 ng/mL) exposure but are increased by ZnSO$_4$ (18µM) as determined by AAS (D). Intracellular findings were recapitulated by flow cytometry and tracking of Zinpyr-1 fluorescence (E, F). Panel B was quantified relative to GAPDH and presented as cumulative data (mean ± SEM) from 3 experiments. Panel C is representative of 3 experiments. Panels A, D, E and F are single experiments (mean).
Figure 5

A) Graph showing Macrophages at 72 hours.

B) Graph showing % ZIP8 mRNA decrease with knockdown.

C) Graph showing % ZIP8 protein decrease with knockdown.

D) Graph showing Zinc (ppm) levels.

E) Flow cytometry showing % of Max FITC-A with different conditions.

F) Flow cytometry showing % of Max MFI with different conditions.
Figure 6. Zinc and ZIP8 alter p50 and p65 activity. (A) p50 and p65 activities in the nucleus are increased by ZnSO₄ (18µM) and LPS (100 ng/mL for 5, 15, 30 and 60 min) as determined by TransAM ELISA. (B) NF-κB binding protein p105 and subunits p50 and p65 levels are unchanged by ZnSO₄ (18µM) when added to LPS (100 ng/mL) treatment within the first h as determined by Western blot of whole cell lysates. (C) ZIP8 knockdown initially increases p50 and p65 activity in the nucleus in response to LPS (100 ng/mL for 30 min), an effect that is eliminated by ZnSO₄ (18µM) by 1 or 2 h as determined by TransAM ELISA. (D) Total cellular levels of p105 and IκBα are unaffected but p50 and p65 are increased by LPS (100 ng/mL for 30 min) in ZIP8 knockdown compared to control, an effect that is eliminated by ZnSO₄ (18µM) or after 1 or 2 h as determined by Western blot. Panels A and D are representative of 2 donors. Panel B is representative of 3 donors. Panel C are cumulative data (mean ± SEM) from 3 experiments.
Figure 6

A

B

C

D

Treatment | Resting | LPS | LPS + Zn | Resting | LPS | LPS + Zn | Resting | LPS | LPS + Zn | Resting | LPS | LPS + Zn
--- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | ---
siRNA | Ctrl | ZIP8 | Ctrl | ZIP8 | Ctrl | ZIP8 | Ctrl | ZIP8 | Ctrl | ZIP8 | Ctrl | ZIP8
p105 | | | | | | | | | | | | |
p50 | | | | | | | | | | | | |
p65 | | | | | | | | | | | | |
IκBα | | | | | | | | | | | | |
β-actin | | | | | | | | | | | | |
Minutes | 30 | 60 | 120 | 30 | 60 | 120 | 30 | 60 | 120 | 30 | 60 | 120

Fold p50 activity

Fold p65 activity

Fold p50 activity

Fold p65 activity
Table 1. The impact of ZIP8 knockdown on macrophage zinc transporter expression. mRNA expression profile of all ZIPs and ZnTs after ZIP8 knockdown or treatment with a siRNA scrambled control followed by LPS (100 ng/mL for 6, 24 or 48 h). Reduction in ZIP8 is the only major change in ZIP expression and also fluctuation of ZnT1 following ZIP8 knockdown as determined by qRT-PCR relative to GAPDH. Table 1 represents cumulative data from 3 different donors (mean values).
Table 1

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Resting | 6h | 24h | 48h
Hours LPS (100 ng/mL)
3.1 Summary

Micronutrient metabolism plays a critical role in innate immune defense against microbial infection. Macrophages exploit the biochemical characteristics of transition metals in part by manipulating their uptake and trafficking following pathogen recognition. Cation re-distribution from the extracellular compartment into the cytosol in response to infection benefits the host in a number of important ways. It inhibits pathogen growth and survival through deprivation of indispensable micronutrients, generates host protective Fenton-reaction-dependent reactive oxygen species and affords nonspecific inhibition of bacterial protein binding [2, 88, 286]. Importantly, internalized micronutrients also help orchestrate vital signaling pathways [33, 88, 287, 288]. Zinc is an essential micronutrient utilized in host defense. Inadequate zinc nutrition reduces innate immune competence, thereby increasing susceptibility to infectious disease [289]. Levels of systemic zinc are buffered in the extracellular compartment by binding to serum proteins, primarily albumin [27, 290], which further regulate zinc's availability. Human cellular zinc metabolism, which is primarily controlled
by fourteen ZIP (Zrt/Itt-like protein) zinc import proteins and ten cytosolic zinc export proteins (ZnTs), is altered by microbe-initiated activation of innate immune cells [42, 217].

Lipopolysaccharide (LPS) stimulates human macrophage gene transcription following extracellular Toll-like receptor 4 (TLR4) binding and sequential activation of intracellular biochemical signaling cascades. The resulting nuclear localization and activation of a number of transcriptional co-activators and transcription factors including but not limited to NF-κB and C/EBPβ largely determines the inflammatory response to infection [178]. Monocytes and macrophages respond to recognition of LPS by increasing production of the SLC39A8 zinc transporter ZIP8 [66]. ZIP8, which is constitutively expressed in human macrophages [66], is induced through the canonical NF-κB pathway following LPS exposure resulting in translocation of ZIP8 protein to the plasma membrane. ZIP8 activity reduces NF-κB-dependent transcription through inhibition of I kappa-B kinase (IKK) [33, 180] in monocytes. NF-κB is a major transcription factor for production of pro-inflammatory cytokines and chemokines that include but are not limited to tumor necrosis factor alpha (TNFα), interleukin six (IL-6) and interleukin eight (IL-8) [178].

LPS stimulation of human macrophages also induces the immune modulatory cytokine interleukin ten (IL-10) [291-293]. IL-10 production by LPS-stimulated macrophages occurs following phosphorylation of the IKK complex and mitogen-activated protein kinases (MAPKS) including p38 and ERK, that
regulate activation of transcription factors including cAMP response element-binding protein (CREB), activator protein one (AP-1), C/EBPβ, C/EBPδ and NF-κB subunit p50 (p50). Concurrent activation of the transcriptional co-activators CREB-binding protein (CBP) and p300 also enhance the IL-10 response. [294-298].

Macrophages differ significantly from monocytes in their phenotype and function. The metabolic pathways responsible for zinc trafficking during macrophage host defense have only begun to be explored [88, 299]. In response to microbes, macrophages produce both pro-inflammatory cytokines and IL-10 in order to coordinate a localized and balanced response aimed at efficiently eliminating infection while minimizing damage to surrounding tissue. IL-10 production by human macrophages in response to infection is essential for regulating immune responses through both autocrine and paracrine feedback mechanisms [294]. Importantly, IL-10 stimulation of murine and human macrophages significantly reduces production of pro-inflammatory cytokines [292, 293, 300, 301].

Our previous studies characterized a reliable in vitro model of inflammation-dependent macrophage zinc metabolism where LPS impacts ZIP8 expression, zinc import and NF-κB activity. Building on those observations, we sought to determine whether ZIP8-mediated transport of extracellular zinc into human macrophages has the capacity to modulate the balance of pro- and anti-inflammatory cytokines produced in response to LPS. Consequently, we
predicted that deficits in zinc metabolism would alter macrophage function through modulation of zinc-dependent signaling pathways that alter host defense. This avenue of research was based upon the observation that ZIP8 is constitutively expressed in resting macrophages and strikingly elevated following LPS exposure. That response is unique compared to the 13 other known zinc import proteins (Fig 3). The findings in this chapter demonstrate that ZIP8-mediated zinc uptake within hours of LPS stimulation results in reduced IL-10 production and an increase in pro-inflammatory cytokine production in macrophages. During LPS exposure, extracellular zinc concentrations within the physiological range markedly reduced IL-10 mRNA expression and protein release and increased mRNA expression of IL-6 and IL-8. ZIP8 knockdown partially reversed zinc-dependent reduction of IL-10 release but did not impact pro-inflammatory cytokine production. However, ZIP8-dependent zinc also did not substantially impact localization of NF-κB subunits to binding sites on the IL-10 promoter. Not anticipating this result, we turned our attention to other transcription factors central to IL-10 regulation. In doing so we observed that nuclear accumulation and activation of the IL-10 inducing transcription factor C/EBPβ is reduced by zinc supplementation of LPS-exposed macrophages. These studies demonstrate for the first time that zinc regulates LPS-mediated immune activation of human macrophages through ZIP8 by reducing C/EBPβ activity and subsequently IL-10 production. Based on these findings we predict
that macrophage zinc metabolism is important in host defense against pathogens as a regulator of inflammation in local tissue microenvironments.
3.2 Results

**Serum buffers in vitro macrophage zinc-dependent cytokine responses**

Human serum contains a multitude of zinc binding species [27], which decrease circulating free zinc concentrations by several orders of magnitude below total serum content [277]. Those factors also reduce zinc and LPS-induced cytokine release in PBMCs [278]. Here it was determined that LPS-induced macrophage cytokine production is responsive to zinc in a serum-dependent manner. The impact of zinc and serum on LPS-induced macrophage cytokine production was investigated *in vitro* by supplementation of zinc sulfate into RPMI cell culture media (which has no zinc added) that was supplemented with variable serum content. Supplementation of zinc sulfate (20 µM) into serum free media during LPS (100 ng/mL) exposure reduced release of IL-10 and trended toward reduction of TNFα and IL-6 while IL-8 release trended toward an increase (Fig 7A) at 6 hours. Addition of 10% autologous serum to culture media during LPS (100 ng/mL) exposure eliminated zinc-dependent reduction of IL-10 and TNFα (Fig 7B). Cellular zinc deficiency induced by prolonged culture in RPMI containing 20% zinc-depleted autologous serum (Fig 4) did not alter the impact of zinc on LPS-induced cytokine responses in serum free media at 6 hours but did reduce IL-6 release independent of zinc supplementation (Fig 7C). Furthermore, zinc-dependent suppression of cytokine responses is a function of intracellular zinc accumulation as demonstrated by intracellular deposition of zinc using
pyrithione which dramatically reduced LPS-dependent IL-10, TNFα, IL-8 and IL-6 release in RPMI containing 10% autologous serum (Fig 7D).

**Zinc modulates LPS-induced macrophage cytokine production**

The inflammatory profile elicited by LPS-exposed macrophages is dependent in part upon micro-environmental zinc availability and ZIP8-mediated importation. The capability of extracellular zinc to impact cytokine production is predicated largely upon its activity as an intracellular second messenger following transport across the plasma membrane. Accordingly, the next investigation focused on a more physiologically relevant model. Using that model we demonstrated that human macrophages exposed to LPS in low serum media, use extracellular zinc to modify inflammatory balance within the local milieu. Macrophage cytokine expression and release was determined at normal physiological zinc concentrations [302] between 10 µM and 18 µM as well as at a higher concentration (40 µM). MDMs were rested or exposed to LPS (100 ng/mL) and/or zinc sulfate for a period of 6 or 24 hours in media containing 2% autologous human serum, the latter contains negligible levels of zinc (Fig 4A). Zinc co-administration during LPS exposure significantly reduced IL-10 mRNA expression (Fig 8A) and protein release (Fig 9A). The impact of zinc on IL-10 release was highly reproducible among donors. In contrast, expression of TNFα, IL-8 and IL-6 mRNA (Fig 8B, 8C and 8D) and corresponding protein release (Fig 9B, 9C and 9D) was increased by co-administration of zinc with LPS.
Inhibition of macrophage IL-10 release by zinc is ZIP8-dependent

Given the constitutive and highly inducible expression of ZIP8 in macrophages [33], we next sought to determine whether ZIP8 was responsible for the zinc-mediated reduction in IL-10. First, ZIP8 was knocked down in MDMs by transfection with ZIP8 or scramble control siRNA (Fig 5C). The impact of ZIP8 or control knockdown-treated MDMs on cytokine release was determined after 24 hour exposure to LPS (100 ng/mL) with or without zinc sulfate (18 µM) supplementation. ZIP8 knockdown almost completely reversed the zinc-dependent reduction of IL-10 release at 24 hours (Fig 10A) indicating that ZIP8 plays a vital role in the zinc-dependent reduction of macrophage IL-10. Conversely, ZIP8 knockdown did not alter LPS-induced macrophage TNFα, IL-8 and IL-6 release at 24 hours (Fig 10B, 10C and 10D). These results confirm that ZIP8-dependent zinc influx is responsible in part for IL-10 inhibition but does not play a major role in the production of prominent pro-inflammatory cytokines in macrophages.

Zinc inhibition of IL-10 production is NF-κB independent

ZIP8-dependent zinc is responsible for reductions in LPS-induced IL-10 release (Fig 10A). ZIP8 also reduces NF-κB-dependent cytokine release in THP1 cells [33] and NF-κB p50 homodimers are in part responsible for inflammation induced IL-10 production [295]. Based on these findings we next determined the
impact of ZIP8-dependent zinc on LPS-induced NF-κB binding to the IL-10 promoter as determined by chromatin histone immunoprecipitation (ChIP).

Initially four putative p50 binding sites (sites A-D) were identified which most closely resemble the consensus p50 binding sequence 5’-G-G-G-(A/G)-N-(A/T)-N-(T/C)-C-C-C-3’ [303, 304] within ten thousand base pairs before the IL-10 transcriptional start site on chromosome 1 (Table 2). ZIP8 knockdown and/or LPS (100 ng/mL) exposure with or without zinc sulfate (18 µM) supplementation for 1 hour did not induce or alter p50 binding to sites (sites A-D) in macrophages as determined by qRT-PCR of precipitated DNA (Fig 11A). In an effort to enhance the ability of that assay to determine alterations in NF-κB binding, five additional putative binding sites (sites E-I) were identified. One additional site based upon the consensus binding sequence was identified in the 3’ untranslated region (site E) and four more sites (sites F-I) in the upstream promoter region were identified (Table 2) [295, 304-306]. Exposure of macrophages to LPS (100ng/mL) and/or zinc sulfate (18 µM) for one hour did not increase binding of p50 or p65 to any of the selected binding sites (sites A-I) in the IL-10 promoter. Levels of antibody-precipitated NF-κB bound DNA from those sites were similar to negative control IgG and several orders of magnitude below the levels observed when compared to the positive control histone H3 (Fig 11B). These and previous findings (Fig 6) indicate that zinc-dependent reductions in IL-10 are not the result of inhibition of NF-κB transcriptional activity.
Zinc supplementation modulates C/EBPβ nuclear localization and phosphorylation

Our previous work revealed that LPS induces the expression of ZIP8 through NF-κB [33]. The importation of zinc into the cytosol reduces subsequent nuclear localization of NF-κB subunit p65 (p65) through direct zinc binding and inhibition of the upstream IKK-complex [33]. However, these observations were restricted to human monocytes and did not involve macrophage studies. Our current findings reveal a distinct, ZIP8-dependent zinc effect on macrophage cytokine and chemokine production highlighted by a reduction in IL-10 release. In order to elucidate the underlying mechanisms responsible for that effect; we next evaluated the impact of zinc on LPS-inducible kinases and transcription factors known to regulate macrophage IL-10 expression.

Accordingly, we determined the impact of zinc sulfate (18 µM) on the amount and phosphorylation of C/EBPβ, the MAP kinases p38 and ERK as well as p50 in macrophages following LPS (100 ng/mL) exposure. Zinc supplementation during LPS exposure led to oscillation of C/EBPβ nuclear accumulation and phosphorylation that was characterized by a rapid reduction of protein at 5 min, followed by an increase at 15 min, and again, a decrease at 30 min (Fig 12A). This pattern was consistently observed with three different donors. In addition, zinc supplementation caused fluctuation in ERK phosphorylation with an early increase followed by a reduction in ERK phosphorylation at 60 min after
LPS (Fig 12B). In contrast, zinc supplementation minimally impacted p-p38 (Fig 12C) and as previously detailed does not impact p50 (Fig 6B). Based on these observations, we propose that the import of extracellular zinc through ZIP8 leads to a reduction in C/EBPβ-dependent and possibly ERK-dependent IL-10 transcription and protein production. To our knowledge this is the first time that zinc has been shown to reduce nuclear C/EBPβ protein levels and its phosphorylation.
3.3 Discussion

Two vital functions of the innate immune system are to initiate inflammation and redistribute micronutrients in favor of the host. Zinc is an essential micronutrient used in host defense. The zinc importer ZIP8 is uniquely induced through stimulation of the NF-κB pathway by LPS in monocytes and functions to regulate inflammation in a zinc-dependent manner. Herein we determined the impact of zinc metabolism following LPS-induced inflammation in human macrophages. Previous studies using human monocyte cell models have shown that zinc can impact the extent of inflammation but results have varied likely due to differences in the approach used. Specifically, zinc supplementation during LPS exposure can both enhance and inhibit monocyte-derived pro-inflammatory cytokine release depending on the dose and timing of zinc exposure [34, 153]. Both direct depletion and enrichment of intracellular zinc using the zinc chelator TPEN or the membrane permeable molecule pyrithione in concert with zinc, respectively, can reduce TNFα and IL-1β release following LPS exposure in monocytic cells [151]. Furthermore, alterations in serum zinc binding protein concentrations in vitro reduce the impact of both zinc and PAMPs on IL-1β and IFN-γ release by human PBMCs [278]. To our knowledge, prior studies have not examined the extent to which zinc impacts immune function in primary human macrophages. Macrophage cytokine release controls the inflammatory balance within the local tissue environment. Synchronous production of both pro-inflammatory and immune modulatory cytokines such as IL-10 regulates this
balance. Our findings support the idea that macrophages utilize available zinc within distinct tissue compartments to increase local inflammation through elevation of IL-8 and IL-6 as well as reduction of IL-10. In our model, we chose to carefully examine zinc-mediated effects by recapitulating the range of physiologic zinc concentrations encountered in humans and various serum concentrations, thereby evaluating zinc-dependent changes in inflammation that occur in situ. It should be noted that zinc has been shown to directly bind LPS and increase potency in PBMCs [283]. In this human macrophage model, zinc reduced IL-10 expression and release while simultaneously increasing that of other cytokines only in the presence of serum, thereby not supporting a generalizable increase in LPS potency. However, these results also indicate that buffering by serum modulates the impact of zinc on inflammation regardless of cellular zinc status. That effect is likely due to protein speciation by zinc primarily through reversible binding to albumin [277, 307]. Consequently the observed serum dependent zinc effect may be altered by fluctuations in zinc binding protein levels such as in protein energy malnutrition, which reduces levels of albumin that loosely binds zinc and increases levels of α-2-macroglobulin which tightly sequesters zinc [308, 309].

Cumulatively, our findings indicate that mononuclear phagocytes have evolved to use ZIP8-dependent zinc to optimize the host inflammatory response to fit the context in which it is required. However, ZIP8-knockdown does not result in complete reversal of zinc-dependent IL-10 reduction (Fig 10A), which
may be due in part to residual ZIP8 protein following knock down (Fig 5C). Importantly, we first observed zinc-dependent reductions in IL-10 at 6 hours following LPS exposure (Fig 8A and 9A) and prior to LPS-induced increases in ZIP8 protein (Fig 3F). These results indicate that the constitutively present pool of ZIP8 (Fig 3A, 3B and 3F) is sufficient to reduce the initial wave of IL-10 expression that is presumably further suppressed by an induced pool of ZIP8 for durable IL-10 inhibition during prolonged LPS exposure.

C/EBPβ is activated following macrophage recognition of extracellular [310] or intracellular pathogens [311] and is indispensable for macrophage bacterial killing [312]. Consistent with these findings (Fig 12A), C/EBPβ is constitutively present in primary human macrophages [313, 314] and LPS markedly increases C/EBPβ expression [313, 315], protein levels and DNA binding in murine macrophages [316, 317]. C/EBPβ-dependent transcription is essential for macrophage cytokine expression, including IL-10, in response to LPS [168, 318-320]. MAPK signaling is also necessary for LPS-dependent IL-10 induction through C/EBPβ [298]. In murine macrophages, C/EBPβ transcription, DNA binding [317] and cytokine production [321-323] is induced through p-38 kinase. Unexpectedly, zinc-mediated inhibition of C/EBPβ accumulation and phosphorylation in human macrophages is not associated with a decrease in p38 phosphorylation (Fig 12C), which indicates that zinc modulates the activity of a yet to be identified kinase(s) or phosphatase(s) upstream of C/EBPβ in a TLR4-dependent signaling pathway. ERK is also important to macrophage pro-
inflammatory cytokine [324] and IL-10 production [294, 296, 297]. Consistent with these findings (Fig 12B), zinc has been shown to inhibit ERK phosphorylation in airway smooth muscle cells [325]. Myeloid cell IL-10 production is proportional to the level of ERK activation [326]. Specifically, LPS induces ERK phosphorylation through activation of the IKK complex in the canonical NF-κB pathway. However, in murine macrophages LPS-induced DNA binding of C/EBPβ, which is reduced by MAPK inhibition is independent of NF-κB signaling [298] and overexpression of C/EBPβ did not increase p50-mediated IL-10 transcription [295]. In contrast to our previous findings in monocytes and cell lines [33] and in support of previous findings in the MDM model (Fig 6), ZIP8-dependent zinc does not modulate p50 or p65 binding on the IL-10 promoter (Fig 11A and 11B). This research supports a macrophage model of zinc-inhibitable, p50-independent induction of IL-10 by C/EBPβ following LPS exposure (Fig 11A, 11B and 12A) that is regulated through ZIP8, which is induced through NF-κB (Fig 13).

Although pro-inflammatory cytokines including IL-6 and TNFα are induced by C/EBPβ, their mRNA levels did not decrease along with the zinc-dependent reduction of C/EBPβ (Fig 7B, 7C, 7D and 12A). C/EBPβ availability in part determines its activity [327], such that zinc-dependent alteration of its abundance may alter cytokine expression independently. Furthermore, phosphorylation of C/EBPβ can be activating or inhibitory and is modified by zinc supplementation (Fig 12A), which may help to explain the differential impact of zinc on pro-inflammatory cytokines and IL-10. The observation that zinc synergistically
induces p50 and p65 activity in macrophages exposed to LPS in a ZIP8 independent manner (Fig 6), combined with the observation that zinc induces the NF-κB dependent cytokines TNFα, IL-8 and IL-6 indicates that macrophage zinc supplementation increases pro-inflammation in a ZIP8-independent manner through activation of NF-κB. Furthermore, zinc increased LPS-induced ERK phosphorylation at 15 min (Fig 12B), which may also in part account for subsequent zinc-dependent increases in pro-inflammatory cytokine expression (Fig 8B, 8C and 8D). Our findings obtained in macrophages are also consistent with previous work that identified both “early” zinc-mediated signaling events (seconds to minutes) and “late” signaling events (minutes to hours), which are both critical for effective signal transmission during pathogen recognition [151, 328]. In this regard our data indicate that ZIP8 may play a role in both signaling phases in macrophages since it is both constitutively present and highly inducible.

Zinc is unique relative to other micronutrients because of its multifaceted impact on cellular function. It is broadly essential for proper protein structure, catalytic function [329] and protection from oxidant damage [330] but is also utilized in specific signal transduction pathways as a tightly regulated second messenger [30, 33, 155]. Given that monocytes and macrophages are in different tissue compartments and produce distinct cytokine and chemokine profiles, it is to be expected that they use zinc to regulate inflammatory responses differently. Moreover, our findings suggest that innate immune host defense mediated by
human macrophages is most likely dysregulated in the setting of zinc deficiency. In this context, IL-10 serves as a negative feedback regulator of TNFα, IL-6, IL-8, proIL-1β and IL-12 [292, 293, 300, 301, 331] production. Accordingly, we predict that significant alteration in macrophage IL-10 release in the setting of zinc deficiency during the initial response to pathogens has the potential to adversely impact both local and systemic inflammation and thereby alter the clinical course of infection.

In summary, we show that extracellular zinc reduces human macrophage IL-10 production following LPS exposure. Most striking, the zinc-dependent effect on IL-10 is facilitated in large part by the zinc transporter ZIP8, which is both constitutively expressed and uniquely induced by LPS. Zinc bio-redistribution into the cytosol following ZIP8 importation leads to an immediate alteration in C/EBPβ nuclear accumulation and phosphorylation as well as an eventual reduction in ERK phosphorylation through mechanisms that remain to be fully defined. Based on our findings, we speculate that ZIP8-mediated reduction of IL-10 and zinc dependent increase in TNFα, IL-6, and IL-8 by tissue macrophages may serve to facilitate more rapid resolution of infection and limit bacterial dissemination. We believe that the fundamental observations made herein have important implications for innate immune defense, particularly against intracellular bacterial pathogens that manipulate IL-10 production and also underscore the importance of proper zinc nutrition to optimize protective immune responses to infection.
3.4 Materials and methods

Reagents

TRIzol, RPMI 1640 with L-glutamine, and DPBS were purchased from Invitrogen (Carlsbad, CA). Zinc sulfate heptahydrate, LPS L-439, pyrithione and BSA were purchased from Sigma-Aldrich (St. Louis, MO). Ficoll-Paque and TMB substrate were purchased from GE Healthcare (Little Chalfont, UK). Rabbit polyclonal antiserum anti-peptide to amino acid residues 225-243 of human ZIP8 (1:1000) was purchased from Covance (Princeton, NJ). Mouse anti-human monoclonal β-Actin (#69101) (1:10,000) antibody was purchased from MP Biomedicals (Santa Ana, CA). Rabbit anti-human monoclonal Lamin B1 (#377001) (1:1000) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-human monoclonal C/EBP β (#1479-1) (1:1000) antibody was purchased from Epitomics (Burlingame, CA). Rabbit anti-human polyclonal ERK (#4695) (1:1000), polyclonal p-ERK (#9101) (1:1000), polyclonal p38 (#9212) (1:2000), monoclonal p-p38 (#9215) (1:2000) and polyclonal p-C/EBP β (#3084) (1:1000) antibodies were purchased from Cell Signaling Technology (Danvers, MA). ZIP8 epitope specific, 21mer small interfering RNA (siRNA) (target sequence TAGGACTTAGGAAATAAATAA) and scramble control siRNA were purchased from QIAGEN (Hilden, DEU).
Human monocyte-derived-macrophage zinc supplementation model

Human monocyte-derived macrophages (MDMs) were isolated and differentiated from PBMCs as previously described [284] (see above). Autologous serum was incubated overnight at 4°C with rocking in Chelex-100 resin (BioRad). MDM monolayers were cultured in RPMI with 20% autologous zinc normal or zinc depleted and cultured 7 days. MDM monolayers were washed and either first incubated with 10µM Pyrithione complexed zinc in RPMI for 30 min, then washed or simply supplemented with warm RPMI or RPMI containing 2 or 10% autologous serum, then supplemented with or without 10, 18, 20 or 40 µM zinc sulfate, with or without LPS 100 ng/mL.

Cytokine assay

Cell supernatants were collected from MDM monolayers at 6 and 24 h.

Supernatants were centrifuged at 1000 × g at 4°C for 10 min to remove cells. Cell free supernatants were then used to measure IL-6, IL-8, IL-10 and TNFα by ELISA per manufacturer’s instructions (R&D Systems).

Protein lysate preparation and Western blot

MDM monolayers were lysed with TN1 buffer to generate whole cell lysate, then incubated at 4°C for 10 min. Lysates were centrifuged at 17,949 × g at 4°C to remove cell debris. To generate nuclear isolates, MDM monolayers were lysed and processed with NE-PER nuclear and cytoplasmic extraction reagents.
(ThermoFisher Scientific) per manufacturer’s instructions. Protein concentration was measured using the Pierce BCA-protein assay kit (ThermoFisher Scientific) per manufacturer’s instructions. Lysates were reduced, denatured and separated by SDS-PAGE, then transferred onto nitrocellulose membranes and blocked with 5% milk in TBS-T, then probed with primary and secondary antibodies of interest and by development using ECL (GE Healthcare). Band densitometry was measured using Image J software. Intensity was determined by subtracting background intensity compared to β-actin or Lamin B1.

Quantitative Real Time RT-PCR
MDM monolayers were lysed with TRIzol (Invitrogen). RNA was isolated using chloroform extraction and ethanol precipitation then converted into cDNA using the ThermoScript RT-PCR system (Invitrogen). Quantitative PCR was performed using SYBR Green (Applied Biosystems). Genes of interest were normalized to GAPDH. Relative copy number (RCN) was determined using the formula: RCN = 2^{-ΔCt} \times 100, where ΔCt is the Ct_{target} − Ct_{reference}. Fold change was calculated by comparing treatment groups to resting controls.

Transfection of MDMs
Following differentiation, PBMCs containing MDMs were transfected with 50 nM ZIP8 or control siRNA using the Amaxa Nulceofector (Lonza) [285] as directed by the manufacturer. Following transfection PBMCs were seeded onto tissue culture
plates and incubated in RPMI-1640 with 10% autologous serum for 2 h at 37°C in 5% CO₂. Lymphocytes were removed by washing with warm RPMI-1640. Adhered, transfected MDMs were repleted with RPMI-1640 containing 20% autologous serum and incubated overnight at 37°C in 5% CO₂ to allow recovery before further treatment.

**Chromatin histone immunoprecipitation assay**

Following 1 hour with or without exposure to LPS (100 ng/mL) with or without supplementation with zinc sulfate (18 µM) ChIP assay was performed as previously described [332] with a few modifications. Briefly, 8 x 10⁶ MDMs in two 15 cm tissue culture plates (Falcon) per group were fixed for 10 min with 1% formaldehyde. Fixation was then quenched by addition of .03125 M glycine and incubation in the dark at room temperature with rocking for 5 minutes. Monolayers were then washed twice with ice cold DPBS scraped into microcentrifuge tubes on ice in DPBS containing protease inhibitor cocktail. MDMs were then pelleted by centrifugation at 1000 x g for 10 min and re-suspended in Lysis buffer and sonicated using a Covaris S series ultrasonicator. Sheered chromatin was then diluted and immunoprecipitated overnight with rotation at 4°C using specific antibodies. Antibodies were pulled down using Protein A-Sepharose beads, which were then washed sequentially with low-salt buffer, high-salt buffer, LiCl buffer, and TE buffer. Protein-DNA complexes were eluted and the crosslinking was reversed at 65°C for 16 h. DNA fragments were
purified and analyzed by qRT-PCR and normalized as percent enrichment relative to un-precipitated input controls for each sample.

Statistics

Each experiment was conducted a minimum of 3 times with different donors. Prism-5 software (Version 5.04; GraphPad) was used to determine the statistical significance of differences in means using one-sided ANOVA with Tukey’s post-test. \( p \) values < 0.05 were considered significant.
Figure 7. Serum buffers zinc inhibition of macrophage cytokine responses.

Zinc (20 µM) co-administration during LPS (100 ng/mL) stimulation of macrophages (A) decreases IL-10 and trends toward a decrease in TNFα or IL-6 and an increase in IL-8 in serum free media but (B) does not alter IL-10 or TNFα protein release in media containing 10% autologous serum at 6 h as determined by ELISA. (C) Macrophage cellular zinc deficiency does not alter the impact of zinc on LPS induced IL-10, TNFα or IL-8, but does reduce IL-6 release. (D) IL-10, TNFα, IL-8 and IL-6 release are inhibited by pyrithione bound zinc (10 µM) in media containing 10% autologous serum. Panel A, C and D are cumulative data from 3 different donors (mean ± SEM; **p < 0.01; ***p < 0.001). Panel B is a single experiment.
Figure 8. Zinc supplementation reduces macrophage IL-10 expression in response to LPS. Zinc co-administration during LPS (100 ng/mL) stimulation of macrophages at 6 h decreases mRNA expression of (A) IL-10 mRNA (as expressed by relative copy number (RCN) on the left and as % change from LPS treatment only on the right side) but increases (B) IL-6, (C) IL-8, and (D) TNFα mRNA expression as determined by qRT-PCR (experimental groups quantified relative to GAPDH). Panels A, B, C and D are representative experiments (white bars) conducted in triplicate or cumulative data (black bars) from 3 different donors (mean ± SEM; *p < 0.05; **p < 0.01; ***p < 0.001).
Figure 8

A

B

C

D

122
Figure 9. Zinc supplementation reduces LPS-induced macrophage IL-10 release. Zinc co-administration during LPS (100 ng/mL) stimulation of macrophages in RPMI + 2% autologous serum decreases IL-10 (A) and trends toward an increase in TNFα (B), IL-8 (C) or IL-6 (D) protein release at 6 h and 24 h as determined by ELISA. Panel A-D are cumulative data from 4 different donors (mean ± SEM; **p < 0.01; ***p < 0.001).
Figure 9

A

% IL-10 relative to LPS

LPS | LPS+10 | LPS+18 | LPS+40 | Resting | Zn18 | Zn40

6 Hours (LPS)

% IL-10 relative to LPS

24 Hours (LPS)

B

% TNFα relative to LPS

LPS | LPS+10 | LPS+18 | LPS+40 | Resting | Zn18 | Zn40

6 Hours (LPS)

% TNFα relative to LPS

24 Hours (LPS)

C

% IL-8 relative to LPS

LPS | LPS+10 | LPS+18 | LPS+40 | Resting | Zn18 | Zn40

6 Hours (LPS)

% IL-8 relative to LPS

24 Hours (LPS)

D

% IL-6 relative to LPS

LPS | LPS+10 | LPS+18 | LPS+40 | Resting | Zn18 | Zn40

6 Hours (LPS)

% IL-6 relative to LPS

24 Hours (LPS)
Figure 10. Reduction in macrophage IL-10 production by zinc is ZIP8-dependent. (A) Zinc-dependent reduction in macrophage IL-10 secretion requires ZIP8, but ZIP8 does not impact TNFα (B), IL-8 (C) or IL-6 (D) release as determined by ELISA of cell free supernatants following ZIP8 knockdown or scramble control and 24 h co-stimulation with LPS (100 ng/mL) and ZnSO₄ (18 µM). Panel A is a representative experiment conducted in triplicate (mean ± SD; *p < 0.05) (on the left) or cumulative data (on the right) from 3 different donors (mean ± SEM; ***p < 0.001; ns = not significant). Panels B, C and D are a single experiment.
Figure 10

A

\[ \text{siScramble Control} \quad \text{siZIP8 Knockdown} \]

IL-10 (pg/mL)

0 50 100 150 200

Resting Zn 18uM LPS Zn 18uM LPS Zn 18uM LPS

24 Hours (LPS)

% IL-10 relative to LPS

LPS LPS+Zn 18uM Resting Zn 18uM LPS LPS+Zn 18uM Resting Zn 18uM

B

\[ \text{siScramble Control} \quad \text{siZIP8 Knockdown} \]

TGF-\(\alpha\) (pg/mL)

0 1000 2000 3000

Resting Zn 18uM LPS Zn 18uM LPS Zn 18uM LPS

C

\[ \text{siScramble Control} \quad \text{siZIP8 Knockdown} \]

IL-6 (pg/mL)

0 10 20 30

Resting Zn 18uM LPS Zn 18uM LPS Zn 18uM LPS

D

\[ \text{siScramble Control} \quad \text{siZIP8 Knockdown} \]

IL-8 (pg/mL)

0 5000 10000 15000 20000

Resting Zn 18uM LPS Zn 18uM LPS Zn 18uM LPS
Figure 11. Zinc-dependent IL-10 inhibition is independent of NF-κB. Zinc or LPS does not alter NF-κB binding to consensus p50 and p65 binding sites on the IL-10 promoter (A) with ZIP8 knockdown or (B) in non-transfected macrophages after LPS (100 ng/mL) exposure and zinc sulfate supplementation (18 µM) for one hour. Panel A is a single experiment. Panel B is cumulative data from 3 experiments (mean ± SEM).
Figure 11

A

IL-10 p50 binding site A

- siScramble
- siZIP8
- IgG
- Histone H3

% of Input

Resting Zn 18uM LPS LPS+Zn18uM

IL-10 p50 binding site B

- siScramble
- siZIP8
- IgG
- Histone H3

% of Input

Resting Zn 18uM LPS LPS+Zn18uM

IL-10 p50 binding site C

- siScramble
- siZIP8
- IgG
- Histone H3

% of Input

Resting Zn 18uM LPS LPS+Zn18uM

IL-10 p50 binding site D

- siScramble
- siZIP8
- IgG
- Histone H3

% of Input

Resting Zn 18uM LPS LPS+Zn18uM

B

IL-10 NF-κB binding site A

- p50
- p65
- IgG
- Histone H3

% of Input

Resting Zn 18uM LPS LPS+Zn18uM - Control + Control

IL-10 NF-κB binding site B

- p50
- p65
- IgG
- Histone H3

% of Input

Resting Zn 18uM LPS LPS+Zn18uM - Control + Control

IL-10 NF-κB binding site C

- p50
- p65
- IgG
- Histone H3

% of Input

Resting Zn 18uM LPS LPS+Zn18uM - Control + Control

IL-10 NF-κB binding site D

- p50
- p65
- IgG
- Histone H3

% of Input

Resting Zn 18uM LPS LPS+Zn18uM - Control + Control

IL-10 NF-κB binding site E

- p50
- p65
- IgG
- Histone H3

% of Input

Resting Zn 18uM LPS LPS+Zn18uM - Control + Control

IL-10 NF-κB binding site F

- p50
- p65
- IgG
- Histone H3

% of Input

Resting Zn 18uM LPS LPS+Zn18uM - Control + Control

IL-10 NF-κB binding site G

- p50
- p65
- IgG
- Histone H3

% of Input

Resting Zn 18uM LPS LPS+Zn18uM - Control + Control

IL-10 NF-κB binding site H

- p50
- p65
- IgG
- Histone H3

% of Input

Resting Zn 18uM LPS LPS+Zn18uM - Control + Control

IL-10 NF-κB binding site I

- p50
- p65
- IgG
- Histone H3

% of Input

Resting Zn 18uM LPS LPS+Zn18uM - Control + Control
Figure 12. Zinc inhibits LPS-induced C/EBPβ. Zinc alters (A) C/EBPβ nuclear accumulation and phosphorylation beginning at 5 min and out to at least 30 min as determined by Western analysis of nuclear extracts and also reduces (B) ERK phosphorylation at 60 min, but does not significantly alter (C) p38 phosphorylation, as determined by Western analysis of whole cell lysates, following 5, 15, 30 or sixty min co-stimulation with LPS (100 ng/mL) and ZnSO₄ (18 µM). Panels A, B and C are representative of 3 experiments. Panels A and B are paired with densitometry.
Figure 12

A

LPS  -  +  -  +  -  +  -  +  +  -  +  -  +  +  +  -  -  -
Zinc +  -  +  -  +  -  +  -  +  -  +  -  +  -  -  +  +  +
P-C/EBP β →
C/EBP β →
Lamin B1 →

5m  15m  30m  60m

B

LPS  -  +  -  +  -  +  -  +  +  -  +  -  +  +  +  -  -  -
Zinc +  -  +  -  +  -  +  -  +  -  +  -  +  -  -  +  +  +
P-ERK →
ERK →

5m  15m  30m  60m

C

LPS  -  +  -  +  -  +  -  +  +  -  +  -  +  +  +  -  -  -
Zinc +  -  +  -  +  -  +  -  +  -  +  -  +  -  -  +  +  +
P-p38 →
p38 →

5m  15m  30m  60m
Figure 13. Graphical abstract of macrophage zinc-dependent inhibition of LPS-induced C/EBPβ-driven IL-10 production. ZIP8-dependent zinc reduces C/EBPβ-driven transcription of IL-10 following LPS activation of macrophage TLR4 signaling by bacterial LPS, which induces further ZIP8 production through NF-κB.
Figure 13
Table 2. Select NF-κB binding sites of IL-10. 9 putative binding sites (sites A-I) were identified within 10 Kbp before or after the IL-10 transcriptional start site based upon the consensus binding sequence and pertinent literature.
Table 2

<table>
<thead>
<tr>
<th>Binding site</th>
<th>Region</th>
<th>bp from start site</th>
<th>Sequence</th>
<th>Resource</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>Promoter</td>
<td>-394</td>
<td>GGGGGACC</td>
<td>Chen et al. Nature. 1998</td>
</tr>
<tr>
<td>D</td>
<td>5’ UTR</td>
<td>-16</td>
<td>GGGGGA</td>
<td>Chen et al. Nature. 1998</td>
</tr>
<tr>
<td>E</td>
<td>3’ UTR</td>
<td>+4378</td>
<td>GGGATCC</td>
<td>Chen et al. Nature. 1998</td>
</tr>
</tbody>
</table>
MACROPHAGE ZINC METABOLISM MODULATES HOST DEFENSE AGAINST MYCOBACTERIUM TUBERCULOSIS

4.1 Summary

Tuberculosis (TB) is a major global cause of morbidity and mortality. Over 2.5 billion people are infected with the pathogen responsible for TB, *Mycobacterium tuberculosis* (*M.tbc*). Initial infection typically results in clinical latency in a healthy host where *M.tbc* resides within macrophages contained inside of host granulomas. However if immune control is compromised, it may reemerge as an active pneumonia that is contagious by airborne transmission and potentially fatal. TB treatment is limited by a lack of universal availability of effective medications, drug toxicity and lack of compliance, the latter resulting in an increasing incidence of multiple drug resistant strains. This is further complicated because individuals with chronically depressed immunity have a much higher risk of developing TB disease [221]. The vast majority of TB infections occur in impoverished nations, where dietary deficiencies are commonplace. Malnutrition leads to immune deficiency thereby increasing susceptibility to infectious pathogens [121, 124]. Lack of adequate zinc can independently manifest many of the immune deficiencies attributed to
malnutrition [134, 137, 141]. Zinc is an essential micronutrient, deficient in the diets of many indigent populations due to reduced access to foods rich in absorbable zinc [93]. Zinc deficiency is a major cause of immune dysfunction and infection globally [93]. *M. tb* infection influences human zinc metabolism. Serum zinc concentrations are significantly reduced in patients with active TB disease [333] and cytosolic zinc concentrations within macrophages significantly increase following infection with *M. tb* [214]. In this context it is well known that infection redistributes micronutrients such as iron [192] and zinc [81] from extracellular to intracellular, which serves as a fundamental host defense strategy against extracellular pathogens that require these nutrients. In the case of iron that redistribution increases host susceptibility to TB [204]. However, it is not yet known whether, and to what extent, zinc bio-distribution influences an intracellular pathogen like *M. tb*. Expression of the zinc importer ZIP8 is rapidly increased in monocytes in critically ill patients and in animal models in response to infection. That increase in ZIP8 results in a reduction of plasma zinc concentrations and an increase in bioavailable, intracellular zinc content [75]. ZIP8 was serendipitously discovered as a result of its induction in monocytes and macrophages in response to BCG infection [66]. Based on this observation, our group discovered that ZIP8 expression is induced in an NF-κB-dependent manner [33]. Infection of human macrophages with *M. tb* modulates zinc metabolism by altering expression of zinc binding metallothioniens and the zinc
exporter ZnT1 as well as increasing phagosomal zinc concentrations, which alter critical zinc responses in *M.tb* [55].

Here we show that the incidence of TB [221] is positively correlated with risk for zinc deficiency [93] on a global scale. Further, critical evaluation of clinical literature reveals that there is a precipitous reduction in circulating zinc levels during active TB infection. Furthermore, *in vitro* ZIP8 expression and protein production increases in human macrophages infected with virulent *M.tb* or BCG. Strikingly, the ZIP8 response is unique in comparison to the other 23 known zinc transporters. Altered expression of ZIP8 corresponds to a redistribution and increase in cytosolic zinc within hours following *M.tb* or BCG infection and supplementation. Supplementation of zinc during macrophage infection leads to reduced IL-10 production and *M.tb* growth. However, ZIP8 itself is not responsible for reducing *M.tb* growth regardless of zinc concentration. Finally, in a novel *in vivo* murine myeloid knockout model, ZIP8 impacts expression of some cytokines and select modulators of adhesion, reactive oxygen species and zinc binding within alveolar macrophages during *M.tb* infection. Further, ZIP8 does not alter released cytokines within the lung during infection. Surprisingly the presence of ZIP8 is associated with increased *M.tb* growth. These novel findings support the general hypothesis that zinc metabolism modulates macrophage antimicrobial responses to *M.tb* through alterations in zinc trafficking. Specifically *M.tb* infection of macrophages induces the expression of ZIP8 and increases in
cytosolic zinc concentrations reduce IL-10 production, which alters host defense against *M.tb* infection.
4.2 Results

TB and zinc status are associated

Zinc deficiency increases susceptibility to bacterial infection [116, 334] and causes immune dysfunction [141]. Compromised immune function is a major predisposing factor of TB susceptibility [221]. Evaluation of decades of peer reviewed clinical publications (1970 – 2011) investigating the impact of TB infection on circulating zinc levels in serum and plasma by methods including dithizone titration, AAS, atomic emission spectroscopy (AES) and mass spectrometry (MS) confirms that active TB patients have reduced circulating levels of zinc (Table 3). Furthermore, zinc deficiency and TB incidence are associated. Comparison of global population data of zinc deficiency risk (as determined by international estimates of dietary zinc intake, serum zinc concentrations and rates of childhood stunting) from the International Zinc Nutrition Consultative Group [93] and rates of TB incidence from the World Health Organization [221] reveals a positive epidemiological correlation (Fig.13A and 13B).

*M.t.* induces ZIP8 production in human macrophages

In light of these and previously described observations, experiments were conducted to determine whether the expression of zinc transporters is altered in human macrophages during infection with virulent *M.t.*. Infection of macrophages with *M.t.* (MOI 5:1) alters the expression of zinc transporters and...
specifically results in induction of ZIP8 expression but not any of the other 23 known zinc transporters (Fig 15A). *M.tbc* and BCG infection of MDMs (MOI 5:1) significantly induces the expression of ZIP8 mRNA (Fig 15B) and protein production (Fig 15C and 15D). Consistent with previously published results [68], ZIP8 induction results in the production of a membrane bound, glycosylated, 140 kDa protein. These findings verify that there is a significant and specific up-regulation of ZIP8 biosynthesis by macrophages following *M.tbc* infection. Similar to *M.tbc* infection (MOI 5:1), ZIP8 protein is increased in macrophages following exposure to the NOD2 agonist muramyl dipeptide (MDP) (5µg/mL) or its *M.tbc* produced N-glycolyl form (GMDP) (5µg/mL) [335] (Fig 15D).

**Mycobacterial infection alters macrophage zinc distribution**

Infection by intracellular pathogens dramatically impacts cytosolic trace element availability and distribution. Fluctuations in the intracellular distribution of iron, copper and zinc are critical host defense responses to mycobacterial infection [55, 212, 266, 267]. However, *M.tbc* counters many of these effects with a repertoire of metal responsive virulence factors [55, 336]. Accordingly we conducted experiments in order to identify the impact of mycobacterial infection on macrophage zinc status in the MDM model. The relative level and subcellular distribution of zinc in macrophages following infection with BCG (MOI 5:1) or *M.tbc* (MOI 5:1) in zinc supplemented (20 µM) conditions were determined using the zinc specific fluorophore Zinpyr-1. We demonstrate that zinc distribution is
altered by mycobacterial infection, which results in part from the import of extracellular zinc. Compared to control cells and cells cultured with either zinc or BCG alone, macrophages incubated with BCG in the presence of zinc display a large number of punctate zinc-specific inclusions evocative of intracellular vesicles (Fig 16A). Importantly, infection with virulent \textit{M}.\textit{tb} also leads to import, redistribution and concentration of intracellular zinc into large punctate inclusions, which are increased in intensity by zinc supplementation (Fig 16B). These findings support the general hypothesis that mycobacterial infection increases zinc uptake and alters its distribution through modulation of macrophage zinc metabolism.

\textbf{Zinc reduces IL-10 production and \textit{M}.\textit{tb} growth in infected macrophages}

IL-10 is an important immune modulator during \textit{M}.\textit{tb} infection. It is associated with TB progression [337] and reactivation [338] in murine models and reduced macrophage host defense capabilities [339-341]. Based on our previous observations that macrophages use zinc to inhibit IL-10 in response to LPS, we conducted experiments to determine the impact of zinc on IL-10 production during \textit{M}.\textit{tb} infection. Supplementation of zinc at the upper end of the physiological range (18 µM) reduces IL-10 release at 24 hours following infection with virulent \textit{M}.\textit{tb} (MOI 1:1) (Fig 17A). Next, based on the fact that IL-10 enhances \textit{M}.\textit{tb} growth and survival within human macrophages [340], we determined the impact of zinc supplementation on \textit{M}.\textit{tb} growth. Zinc
supplementation is associated with a reduction of *M.tb* growth as measured by light emission from an established bioluminescent strain of virulent *M.tb* [342] at 72 hours post infection (MOI 1:1) (Fig 17B), presumably as a result of IL-10 inhibition.

**ZIP8 does not impact *M.tb* growth in human macrophages**

Host defense strategies of trace element redistribution can result in reduced microbial growth. Manipulation of intracellular zinc through altered zinc transporter expression impacts growth of intracellular fungal [88, 217] and bacterial [219] pathogens. Having observed a zinc-dependent inhibition of *M.tb* growth within macrophages along with an increase in ZIP8 production, we performed experiments to determine the impact of ZIP8 and ZIP8-dependent zinc flux on *M.tb* growth. The impact of ZIP8 on *M.tb* infection of macrophages was determined using siRNA-mediated ZIP8 knockdown. ZIP8 knockdown of 60% was durable through 72 hours post infection with an 80% knockdown achieved at 24 hours (Fig 18A). Following knockdown and infection with virulent *M.tb* H₃₇R₅ (MOI 1:1) we revealed that ZIP8 does not impact mycobacterial growth by CFU assay at 2, 24, 48 or 72 hours post infection (Fig 18B). In order to further characterize any potential zinc-dependent impact of ZIP8 on *M.tb* growth, ZIP8 knockdown or scramble control macrophages were infected with the luciferase-producing *M.tb* strain (MOI 1:1) during zinc supplementation (18µM). Over the course of five days, under those conditions, ZIP8 does not impact growth of *M.tb*.
within human macrophages; however a small trend did emerge at later time points for a ZIP8-dependent increase in *M.tbc* growth during zinc supplementation (Fig 18C).

**ZIP8 minimally impacts *M.tbc*-induced inflammatory mRNA expression by pulmonary murine macrophages in vivo**

ZIP8 has been shown to alter cytokine signaling in multiple human myeloid cell types including monocytes, macrophages and in T-cells in response to immune activation [30, 33]. It is also highly inducible in response to infection *in vivo* [33, 74, 75]. We conducted experiments to evaluate the potential impact of ZIP8 on TB disease using a novel *in vivo* murine model of myeloid specific ZIP8 knockout (data not shown) developed by the Knoell Lab group. ZIP8 knockout and control mice received a mid-dose infection (~1000 bacilli/mouse) with virulent *M.tbc H*37R*<i>v</i> by aerosolization and then lungs were harvested at 22 days. Lung tissue macrophages and alveolar macrophage mRNA expression profiles were then determined. Expression was evaluated for cytokines including: IL-1β, TNFα, IL-10, IL-6, IL-4, IL-13, the adhesion molecule ICAM and the modulators of free radical formation, detoxification and intracellular zinc buffering iNOS, manganese super oxide dismutase (MnSOD), and MT1 or MT2, respectively. *M.tbc*-induced expression of IL-1β, TNFα, ICAM and MnSOD is reduced in ZIP8 knockout alveolar macrophages (Fig 19A). These results indicate that ZIP8 increases pro-inflammatory signaling, migration and free radical detoxification in *M.tbc*-infected
alveolar macrophages. However, ZIP8 did not significantly impact whole lung tissue macrophage mRNA expression of these mediators in response to *M. tb* (Fig 19B).

**ZIP8 does not impact pulmonary cytokine release and may increase *M. tb* growth in vivo**

Based upon our observation that ZIP8 promotes pro-inflammatory cytokine expression in alveolar macrophages *in vivo*, which is associated with macrophage host defense against *M. tb* [230], we next evaluated ZIP8 dependent cytokine release *in vivo*. We assayed cytokine levels including: IL-1β, TNFα, IL-6, IL-2, IL-12, IFN-γ and the growth factor GM-CSF in the alveolar microenvironment during *M. tb* infection. ZIP8 knockout does not impact pulmonary cytokine or GM-CSF release into alveolar fluid (Fig 20A). Finally, we evaluated the impact of ZIP8 on *M. tb* growth *in vivo*. We evaluated *M. tb* growth at 22 days post infection in myeloid specific ZIP8 knockout mice compared to mice heterozygous for ZIP8 or wild-type controls. Surprisingly, the presence of ZIP8 resulted in enhanced *M. tb* growth within lung tissue at 22 days, a critical juncture where innate immune defense becomes overshadowed by adaptive immune defense (Fig 20B).
4.3 Discussion

Risk for TB is increased by malnutrition-induced immune deficiency [343] and zinc supplementation is capable of reversing this effect [344]. Accordingly, the observation that zinc deficiency occurs with TB disease (Fig 14A and 14B) is an obvious sequitur in light of decades of research revealing that zinc deficiency disrupts immune function [289]. This disruption likely breaches homeostatic host defense sufficiently to facilitate infection by *M. tb*, a pathogen that thrives in the setting of deficits in immune regulation [239]. The epidemiological association highlights the critical importance of zinc metabolism in host defense against TB. Although zinc deficiency may increase susceptibility to *M. tb* infection and/or reactivation due to immune suppression, most trials evaluating the impact of zinc supplementation as an adjunct treatment to antibiotic therapy have shown zinc to have little to no impact on clinical course and recovery [345-347]. However, the cumulative immune supportive benefits of prolonged zinc sufficiency are likely to be more important in promoting early host defense against infection. Previous studies highlighting reductions in serum zinc concentrations during active TB disease (Table 3) are consistent with a model of re-compartmentalization of zinc out of the vasculature in response to TB as is seen in the acute phase response [81]. Preexisting zinc deficiency may also contribute to those findings.

The increased intracellular sequestration of zinc, similar to iron, is a mechanism of nutritional immunity in which vascular trace element deprivation limits extracellular growth of invading pathogens [2]. ZIP8 is elevated in
circulating peripheral blood monocytes during the acute phase response, which is associated with reduced serum zinc concentrations [75] and may play a significant role in this phenomenon. In addition to induction in *M. tb*-infected macrophages (Fig 15), ZIP8 is up-regulated by inflammatory signaling in uninfected macrophages (Fig 3C) and tissue epithelial cells [68] leading to similar redistribution of zinc out of the extracellular compartment within local tissue environments. Redistribution of zinc out of systemic circulation may negatively impact extracellular pathogens, but similar to iron [203, 204] could have a beneficial impact on the growth of intracellular *M. tb*. Zinc is an essential component of survival for all bacteria [216] and promotes mycobacterial growth [348]. In that context induction of ZIP8 in response to infection (Fig 15) and subsequent zinc import across the plasma membrane into the cytoplasm (Fig 16B) could benefit *M. tb* growth. Therefore, ZIP8-dependent increases in intracellular zinc may contribute to our *in vivo* observations of decreased growth in the lungs of ZIP8 knockout mice (Fig 20B).

ZIP8 is induced through NF-κB (Fig 2A) [33]. Inflammatory stimuli including TNFα (Fig 3C) and LPS (Fig 3F), which induce ZIP8 expression in macrophages, activate NF-κB signaling through extracellular receptors as does *M. tb* [349]. MDP and GMDP, which also induce ZIP8 production (Fig 15D), activate NF-κB through the intracellular receptor NOD2, as does infection by BCG and *M. tb* [285, 350]. Stimulation of macrophages and monocytes with the hyper-inflammatory BCG cell wall cytoskeletal extract that contains ligands for
multiple NF-κB activating receptors also induces ZIP8 [66]. Consequently, inputs into the NF-κB pathway from multiple receptors likely cumulatively result in a significant induction of ZIP8 production in macrophages in response to infection by BCG and *M. tb*. The comparatively greater induction of ZIP8 in response to BCG as compared to *M. tb* (Fig 15B and 15C) is likely due to enhanced activation of NF-κB signaling by BCG relative to *M. tb* [285]. Yet, because ZIP8 is the most highly expressed zinc transporter in resting macrophages (Fig 15A) and is constitutively produced (Fig 15C) it has the potential to modulate zinc-dependent responses regardless of induction through NF-κB. Of note, the zinc-induced transporter ZnT1 is also highly expressed constitutively but contrary to previous reports [55] is not increased in response to *M. tb* infection (Fig 15A).

Levels of intracellular zinc within intracellular vesicles are known to increase in macrophages infected by *M. tb* [214] but changes in the gross cellular distribution of macrophage zinc following mycobacterial infection were previously uncharacterized. Zinpyr-1, a zinc-specific probe, was chosen for our studies because of its unique attributes. Although unbound Zinpyr-1 background fluorescence is relatively high, it does localize to the lysosomal pathway and intracellular vesicles with an emission intensity that is unaffected between the pH range of 5.5-8.0. Furthermore, it produces discernable changes in fluorescence within punctate zinc inclusions in response to changes in intracellular zinc concentration [280]. These properties allowed for the observation of increased zinc concentration (as presumably membrane bound puncta) following infection.
with BCG and to a greater extent *M. tb*; an effect that was intensified by zinc supplementation (16A and 16B). Intracellular zinc distribution is highly regulated, such that zinc is principally sequestered in complex with proteins and/or within organelles [351]. Trafficking of zinc into and out of storage vesicles is accomplished by human zinc transporters including ZIP8 [42]. ZIP8 is present on the plasma membrane and intracellular vesicles [33, 66, 68] and transports zinc across the plasma membrane into cells or out of intracellular vesicles [30, 69]. These properties as well as its high constitutive expression in macrophages make it likely that ZIP8 plays a role in the observed cellular zinc influx following infection. However, it remains unknown whether ZIP8 influences zinc distribution towards or away from the mycobacterial phagosome. Further study of zinc trafficking relative to the localization of ZIP8 and the mycobacterial phagosome in the context of cellular zinc status could reveal whether ZIP8 induction during *M. tb* infection impacts the previously proposed host defense strategy of zinc poisoning during *M. tb* infection [55].

Zinc supplementation was previously shown to skew cytokine signaling toward a pro-inflammatory profile in macrophages responding to LPS, particularly through inhibition of IL-10 production (Fig 8 and Fig 9). This effect is likely a result of C/EBPβ inhibition independent of NF-κB (Fig 6, Fig 11 and Fig 12). Zinc dependent inhibition of IL-10 is recapitulated during *M. tb* infection (Fig 17A). The resulting 30% decrease in IL-10 release is associated with a 25% decrease in *M. tb* growth after 3 days of infection (Fig 17B). The role of IL-10 as a host
susceptibility factor during human *M.tb* infection is well established [239, 341]. Furthermore, zinc supplementation was shown to reduce IL-10 mRNA expression in whole blood of patients with active TB [270]. Elevated IL-10 levels during *M.tb* infection reduce macrophage host defense functions including but not limited to; phagosome-lysosome fusion [340], autophagy [352], pro-inflammatory cytokine responsiveness and production [353], nitric oxide production [341] and antigen presentation [354]. However, it cannot be ruled out that the growth effect is independent of IL-10 reduction and further confirmatory studies should be conducted. The impact of zinc on macrophage host defense functions during *M.tb* infection is an under explored area of research with promising potential for the generation of translationally applicable future findings. Our findings for the first time reveal that modulation of IL-10 by zinc is a critical host defense function that is potentially compromised by zinc deficiency. Advancing these findings may help to further explain the epidemiological association between zinc deficiency and TB incidence.

Based upon the findings in Fig 17 and previous findings showing that reduction of IL-10 by zinc is in part determined by ZIP8 (Fig 10A), we tested the hypothesis that ZIP8 and/or ZIP8-dependent zinc would reduce *M.tb* growth *in vitro* within human macrophages. Unexpectedly, under the conditions studied it did not (Fig 18B and 18C). In transfected macrophages the impact of zinc supplementation on *M.tb* growth in control cells at 72 hours is also absent (Fig 18C). One possibility is that the stresses of siRNA nucleofection in combination
with \textit{M.\textit{tb}} infection may have altered macrophage responsiveness to zinc. In addition, although ZIP8 knockdown was highly reproducible, significant and durable (Fig 18A), it is not 100\% effective. The knockdown may have been insufficient to determine the potential impact of ZIP8 on \textit{M.\textit{tb}} growth \textit{in vitro}. Alternatively, the impact of zinc on \textit{M.\textit{tb}} growth within human macrophages may be independent of ZIP8 in which case exploration of alternative zinc transporters is warranted. We do note that in zinc-supplemented macrophages, ZIP8 knockdown is associated with a trend towards decreased \textit{M.\textit{tb}} growth (Fig 18C). This trend is mirrored \textit{in vivo} by assessing \textit{M.\textit{tb}} growth in ZIP8 knockout mice (Fig 20B), indicating that aspects of ZIP8 function relate to host susceptibility. Paradoxically, ZIP8 knockout reduced TNF\(\alpha\) expression within murine alveolar macrophages (Fig 19A), which is critical to control of infection [246]. One potential explanation for the \textit{in vivo} growth finding is that the presence of ZIP8 within the phagosomal pathway may be important in relieving the stress of zinc toxicity within the \textit{M.\textit{tb}} phagosome. ZIP8 could serve as a direct phagosomal zinc efflux pump and/or influence the expression, activity or localization/dimerization of other phagosomally situated zinc transporters such as ZnT1 through cellular influx of extracellular zinc.

These findings provide insight into the elevated risk for TB associated with zinc deficiency and demonstrate a role for macrophage ZIP8 as a potential modulator of systemic and cellular zinc status. Furthermore, during macrophage infection by \textit{M.\textit{tb}}, which induces ZIP8, zinc is redistributed intracellularly (Fig
This effect is increased by zinc supplementation up to physiological concentrations and is associated with reduced IL-10 levels and impaired *M.tb* growth. However, that zinc effect is independent of ZIP8. ZIP8 may compromise host defense and should be further investigated as a host susceptibility factor. The epidemiological co-occurrence of zinc deficiency and TB is potentially contributed to by loss of zinc-dependent control of IL-10 production. The effect of zinc on IL-10 is likely critical to host defense during the early stages of infection as well as potentially during the latency. Although zinc supplementation during active TB disease has not been proven to be protective, these concepts should be evaluated in the context of preventative therapy. Potential recommendations might include zinc supplementation of high-risk populations such as household contacts of TB patients and known latently infected individuals.
4.4 Materials and methods

Reagents

TRIzol, RPMI 1640 with L-glutamine and DPBS were purchased from Invitrogen (Carlsbad, CA). Zinc sulfate heptahydrate, Zinpyr-1, BSA and MDP were purchased from Sigma-Aldrich (St. Louis, MO). GMDP was purchased from InvivoGen (San Diego, CA). Ficoll-Paque and TMB substrate were purchased from GE Healthcare (Little Chalfont, UK). Rabbit polyclonal antiserum anti-peptide to amino acid residues 225-243 of human ZIP8 (1:1000) was purchased from Covance (Princeton, NJ). Mouse anti-human monoclonal β-Actin (#69101) (1:10,000) antibody was purchased from MP Biomedicals (Santa Ana, CA). ZIP8 epitope specific, 21mer small interfering RNA (siRNA) (target sequence TAGGACTTAGGAAATAAATAA) and scramble control siRNA were purchased from QIAGEN (Hilden, DEU). Agar was prepared with Bacto Middlebrook 7H11 agar, oleic acid-albumin-dextrose-catalase enrichment medium, and glycerol purchased from Difco Laboratories (Detroit, MI).

Isolation, culture and transfection of human macrophages

Isolation, culture and transfection of human macrophages were conducted as described in the preceding text.
Bacterial strains and culture

Lyophilized *M.tbc* H$_{37}$R$_{v}$ (ATCC #25618) and *M. bovis* BCG (ATCC #35734) were obtained from the American Tissue Culture Collection (Manassas, VA). They were reconstituted prior to use as previously described [285]. Acceptable bacterial concentrations of 1–2 × 10$^8$ bacteria/mL and levels of clumping less than 10% were verified by counting on a Petroff-Hausser chamber. The bacteria were greater than 90% viable by CFU assay.

Macrophage infection

Infection was conducted as previously described [350]. Briefly, MDMs were cultured at a density of 4.0 × 10$^5$/mL for 12 days or 6 days for transfected cells were incubated with *M.tbc* or BCG at a multiplicity of infection of 5 to 1 or 1 to 1 in RHH for 30 min at 37°C in 5% CO$_2$ on a platform shaker for bacterial dispersion followed by an additional incubation for 90 min. Monolayers were then washed 3x with RPMI and repleted with RPMI with 2% human autologous serum. For experiments utilizing NOD2 agonists, MDMs were incubated with MDP (5 µg/ml) or GMDP (5 µg/ml) for 24 or 48 h in RPMI containing 2% autologous serum.

*M.tbc* intracellular growth assay in macrophages

Colonial forming unit assay or relative light unit emission luciferase activity assays using infected macrophage lysates were performed in triplicate as previously described [342, 355]. Briefly, MDM monolayers were infected with *M.tbc* H$_{37}$R$_{v}$ or
M.\textit{tb} H\textsubscript{37}R\textsubscript{v}-Lux at a multiplicity of infection of 1 to 1 as described in the preceding text. MDM monolayers were then processed for enumeration of CFU or RLU respectively. For CFU assay colonies were counted following 4 week incubation on 7H11 agar.

**Quantitative Real Time RT-PCR and Western**

Protein lysate preparation, Quantitative Real Time RT-PCR and Western blot were conducted as described in the preceding text.

**Knockout mice**

All animal studies were conducted in accordance with approval from the Ohio State University’s Institutional Animal Care and Use Committee. Mouse studies were performed using 8 week old C57BL/6 Cre-lox ZIP8\textsuperscript{+/−} mice generated by crossing Zip8 flox/flox breeder pairs with Cre-bearing deleter mice in order to obtain myeloid-specific Zip8 deficiency, ZIP8\textsuperscript{+/-} mice or wild-type controls. Conditional deletion of the ZIP8 in myeloid lineage cells was driven by the LysM-Cre promotor system. ZIP8 deletion on exon 3 within two generations was verified by genotyping analysis and confirmed by qRT-PCR. 2 mice were used for each experimental group. \textit{M.tb} H\textsubscript{37}R\textsubscript{v} aerosol-infection with a mid-dose of (1000 bacilli/mouse) was performed within a BSL3 facility. Mice were euthanized by CO\textsubscript{2} asphyxiation in accordance with approved protocols. CFU assay of lung homogenates was conducted by plating serial dilutions as previously described.
Alveolar and whole lung macrophages were collected onto tissue culture plates, isolated and mRNA was collected as previously described.

**Bioplex cytokine assay**

Bronchoalveolar lavage fluid was collected from mice by washing of the lungs with sterile saline (0.9% NaCl) at 22 days post infection with *M. tb* H$_{37}$R$_{v}$ and concentrated by centrifugation at 4°C as previously described [356]. Concentrated alveolar fluid was then evaluated for cytokine levels by Bio-plex assay (Bio-Rad) per the manufacturer's instructions.

**Statistics**

Statistical tests were conducted as described in the preceding text.
**Figure 14. TB incidence is correlated with risk for zinc deficiency.**

Population estimates of TB incidence & zinc deficiency risk were obtained from 149 nations (IZiNCG, 2004; WHO, 2010) (A) Co-occurrence was graphically depicted and then evaluated for potential associations using Chi-Squared analysis. (B) A strong correlation exists between TB incidence and zinc deficiency on a global scale ($\alpha = 0.05$, $p < 0.0001$).
Figure 14

A  Global TB incidence correlates with risk for zinc deficiency

Estimated incidence of all forms of TB per 100,000 population
WHO Global Tuberculosis Control Report 2010

Estimated zinc deficiency risk category
IZINC Assessment of the Risk of Zinc Deficiency in Populations and Options for Its Control 2004

Low Risk  High Risk
Medium Risk  Not Evaluated

Chi-Squared Analysis: P <0.0001

B
Figure 15. ZIP8 is induced by macrophage *M.tb* infection. (A) Relative ZIP and ZnT mRNA expression, as determined by qRT-PCR, is altered in MDMs infected by *M.tb* H₃₇Rᵥ with changes in ZIP8 being > 3x those of any other transporter (n=1). (B) ZIP8 mRNA, as determined by qRT-PCR in MDMs infected by either *M.tb* H₃₇Rᵥ or BCG, exhibited a > 7-fold increase relative to uninfected controls, respectively, within 72 hours of infection (each experimental group in A & B was performed in triplicate and quantified relative to GAPDH) (mean ± SD). (C) ZIP8 protein levels, as determined by Western analysis, increased following *M.tb* H₃₇Rᵥ or BCG infection (n=3). Densitometric analysis of Panel C confirms the increase. (D) ZIP8 protein levels as determined by Western blot analysis are significantly induced by MDP (5µg/mL), GMDP (5µg/mL) and *M.tb* H₃₇Rᵥ infection within 48 hours of exposure or infection (n=2). (A and B were quantified relative to GAPDH).
Figure 15

A

Resting 24 h 48 h 72 h

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<th>48 h</th>
<th>72 h</th>
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B

Fold ZIP8 mRNA

C

ZIP8 Protein (a.u.)

D

ZIP8 → 

β-actin → 

Hours 24 48 72
Figure 16. BCG and *M.tb* infection alters macrophage zinc distribution. The distribution of available intracellular zinc, as determined by fluorescence confocal microscopy of MDMs stained with Zinpyr-1 (5µg/mL) and DAPI, is altered in response to (A) 6 hour infection with *M. bovis* BCG (MOI 5:1) or (B) 4 hour infection with *M.tb* H$_{37}$R$_v$ (MOI 5:1). Zinc-dependent intracellular fluorescence increased in cultures that had 20µM zinc sulfate added into their media following infection indicating cellular uptake of zinc from the extracellular compartment (images are representative of ten z-stack images in panel A or single plane images in panel B from 2 biological replicates per group in 1 experiment each).
Figure 16

A

Resting                             Zinc 20µM

BCG                                   BCG+ Zinc 20µM

B

Resting                             Zinc 20µM

*M.tbc                                *M.tbc+ Zinc 20µM
Figure 17. Zinc supplementation reduces macrophage IL-10 expression and bacterial growth during *M. tb* infection. Zinc co-administration during *M. tb* H₃₇Rᵥ infection (MOI 5:1) of macrophages in RPMI + 2% autologous serum (A) decreases IL-10 at 24 h as determined by ELISA or (B) *M. tb* growth at 72 hours as determined by assay of RLUs from luciferase producing *M. tb* H₃₇Rᵥ-Lux.

Panel A is a representative experiment (white bars) conducted in triplicate or cumulative data (black bars) from 2 different donors (mean ± SD; *p* < 0.05).

Panel B is cumulative data from 3 different donors (mean ± SEM; *p* < 0.05).
Figure 17

A

IL-10 (pg/mL)

Resting  M.tb  M.tb & Zinc

*  

% IL-10 release relative to M.tb

M.tb  M.tb & Zinc  Resting

B

Zn-dependent % change M.tb (RLU)

24  48  72

*  

163
Figure 18. ZIP8 does not impact *M. tb* growth in human macrophages. (A) ZIP8 protein levels as determined by Western analysis are significantly reduced by nucleofection of ZIP8-specific siRNA relative to scramble control siRNA in MDMs infected with *M. tb* H37Rv. Densitometric analysis of panel 2E reveals an 85% knockdown of ZIP8 protein within 24 hours with a durable 60% knockdown of ZIP8 protein through 72 hours. ZIP8 knockdown does not impact *M. tb* growth within MDMs as determined by (B) CFU assay of *M. tb* H37Rv through 72 h or (C) in MDMs cultured in the presence or absence of ZnSO₄ (18µM) as determined by assay of RLUs from luciferase producing *M. tb* H₃₇Rₐₙ₅-Lux through 120 h. Panel A is a representative experiment from 4 different donors. Panels B and C are cumulative data from 3 donors.
Figure 19. ZIP8 alters alveolar macrophage cytokine expression in vivo.

Infection of Cre-lox homozygous myeloid specific ZIP8 knockout C57B/6 mice by mid-dose (100 bacilli/mouse) aerosol infection with *M. tb* H37Rv (A) reduces IL-1β, TNFα, ICAM and MnSOD but only marginally impacts IL-10, IL-6, IL-4, IL-13, iNOS, MT1 and MT2 mRNA expression in alveolar macrophages and (B) does not impact their expression in whole lung tissue macrophages compared to wild-type control as determined by qRT-PCR. Panel A is pooled mRNA from two mice. Panel B is cumulative data from 3 mice (mean). A and B were quantified relative to GAPDH.
Figure 19

A

Alveolar Macrophages

B

Whole Lung Macrophages
Figure 20. ZIP8 marginally impacts pulmonary cytokine release and may increase *M.* _tb_ growth *in vivo*. Infection of Cre-lox homozygous myeloid specific ZIP8 knockout C57B/6 mice by mid-dose (100 bacilli/mouse) aerosol infection with _M.* _tb_ H_{37}R_{v} (A) does not alter levels of IL-1β, TNFα, IL-6, IL-2, IL-12, IFN-γ or GM-CSF in alveolar fluid as determined by bio-plex assay and (B) trend toward a decrease in bacillary load as determined by CFU assay of whole lung homogenate. Panel A are cumulative data from 3 mice. Panel B are cumulative data from two mice in duplicate.
Figure 20

A

- IL-1β (pg/mL)
- TNFα (pg/mL)
- IL-6 (pg/mL)
- IL-2 (pg/mL)
- IL-12 (pg/mL)
- IFN-γ (pg/mL)
- GM-CSF (pg/mL)

B

- Lung Log10 Mtb CFU

WT, Het, KO comparison for cytokine levels and Mtb CFU.
Table 3. Serum zinc concentrations in TB. A search of the Medline online database using the search terms “zinc” “tuberculosis” returned 246 search results. For inclusion, studies were required to have been accessible through the information resources of The Ohio State University and provide statistics of a comparison between the serum or plasma zinc concentrations of a TB-infected group against a control group. 19 studies qualified for inclusion.
### Table 3

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<td>85.47 ± 28.90</td>
<td>54.09 ± 14.16</td>
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<td>&lt; 0.05</td>
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<td>154.92 ± 15.27</td>
<td>75.76 ± 9.38</td>
<td>11</td>
<td>14</td>
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<td>117.13 ± 4.2</td>
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<td>20</td>
<td>20</td>
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<td>65.53 ± 9.8</td>
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<td>Ghulam H, 2009</td>
<td>95.82 ± 3.12</td>
<td>73.56 ± 6.90 (20-29 years)</td>
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<td>94.78 ± 5.02</td>
<td>68.22 ± 5.33 (30-39 years)</td>
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<td>85.23 ± 2.16</td>
<td>53.46 ± 2.96 (50-65 years)</td>
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CHAPTER 5: SYNTHESIS

This dissertation explores the role of zinc metabolism in regulating innate immune responses of macrophages within the context of the macrophage response to infection by the classic intracellular pathogen *M. tb*. It tests the central hypothesis that zinc modulates macrophage host defense during infection with *M. tb* through the zinc transporter ZIP8. The cumulative results demonstrate that *M. tb* infection of macrophages alters zinc metabolism through induction of ZIP8 (Fig 15) and that zinc benefits host defense against *M. tb* (Fig 17B) in part by reducing immune suppressive cytokine signaling (Fig 17A). However, paradoxically, our results indicate that ZIP8 potentially increases *M. tb* growth (Fig 20B). Thus, the relationship between zinc and ZIP8 in macrophages is complex. Additionally, in addressing the question of how zinc metabolism impacts macrophage antimicrobial resistance to *M. tb*, insights into zinc-dependent immunomodulation of human macrophages were concomitantly discovered. In turn these insights provide substrate for additional questions regarding the intricacies of macrophage zinc metabolism in immunity and infectious disease.

ZIP8 production in human macrophages is increased by several NF-κB activating stimuli including infection by *M. tb* and BCG (Fig 15) [285, 357], the
bacterial PAMPs LPS (Fig 3D, 3E and 3F) [358], MDP and GMDP (Fig 15D) [350] as well as the inflammatory cytokine TNFα (Fig 3C) [178]. Determination of the precise receptors, down-stream signaling molecules and transcription factors responsible for *M. tb*-dependent induction of ZIP8 is an important step in understanding the mycobacterial effectors that modulate zinc metabolism. *M. tb* antigens activate multiple cell surface and intracellular receptors, which include but are not limited to the plasma membrane bound receptor TLR4 and the intracellular receptor NOD2 [359]. *M. tb* infection transiently induces NF-κB [285, 360] which is known to induce ZIP8 expression [33] and also to regulate macrophage inflammatory responses [178]. Therefore, *M. tb* may induce ZIP8 through NF-κB by activating both cell surface and intracellular receptors. In that case, ZIP8 induction and function are multiphasic and may be coordinated by *M. tb* throughout the course of macrophage infection from phagocytosis to residence of *M. tb* in the bacterial phagosome. Future studies evaluating mycobacterial antigens such as cell wall mannosylated lipoarabinomannan (ManLAM) [361] or secreted ESAT-6 [362] for their ability to induce ZIP8 may further define the role of ZIP8 in the host microbe interaction.

In addition to inducing ZIP8 expression, *M. tb* infection impacts macrophage zinc metabolism by altering intracellular zinc distribution (Fig 16B) and elevating intraphagosomal zinc concentrations [214]. Expression of zinc binding species including MTs is also increased following *M. tb* infection [55]. ZIP8 is constitutively expressed in resting macrophages and both LPS exposure
and M.tb infection result in a significant induction of ZIP8 within 24 hours that is unique relative to the other 23 zinc transporters (Fig 3, 15 and Table 1). In previous work [33] our group revealed that immediate zinc accumulation in THP1 cells exposed to LPS relies upon ZIP8 influx. Conversely, in human macrophages exposed to LPS for 6 hours intracellular zinc accumulation is independent of ZIP8 (Fig 5D, 5E, 5F). However those findings do not exclude the possibility that transient ZIP8-dependent zinc flux contributes to observed changes in intracellular zinc distribution and concentration during mycobacterial infection (Fig 16B). Posttranslational modification of ZIP8 similar to that observed in ZIP7 [170] is a possible mechanism through which the activity of the constitutive and induced pool of ZIP8 may be modified. Co-immune precipitation and mass spectrometry of ZIP8 in macrophages responding to M.tb infection or exposure to M.tb antigens could reveal candidate protein modifiers. However, in addition to posttranslational modification, localization of the inducible pool of ZIP8 is likely key to its immune modulatory function.

Studies in macrophages investigating the intracellular flux of iron [212] and copper [363] during M.tb infection implicated specific transporters in the increase of phagosomal divalent cation concentrations. During H. capsulatum infection [88] macrophages utilize a strategy of phagosomal zinc deprivation as a mechanism of defense against pathogenic invasion. Similarly a role for ZIP8 as a mechanism of phagosomal zinc-deprivation has been proposed [218], which may be the mechanism facilitated by the M.tb-induced pool. However, we found that
ZIP8 may be a host susceptibility factor (Fig 20B). In that capacity its localization to the *M. tb* phagosome and subsequent zinc efflux activity would likely curtail zinc poisoning. Zinc accumulates within the mycobacterial phagosome at least through 24 hours [214] when ZIP8 protein concentrations are significantly elevated and continue to increase at least through 48 hours (Fig 15C). Validation of the hypothesis that induction of ZIP8 is an *M. tb*-induced susceptibility factor implicated in detoxification of phagosomal zinc poisoning would require a number of experiments. Principal studies should include evaluation of 1) the co-localization of ZIP8 with phagosomal and lysosomal proteins, and 2) the presence of *M. tb* and zinc within the phagosome under zinc sufficient conditions over a continuum of days. That investigation could determine whether ZIP8 exists on the *M. tb* phagosome and whether it functions to reduce intraphagosomal zinc concentrations over the course of its induction by *M. tb*. Those studies could be conducted in our model of cellular zinc deficiency (Fig 4C), which we postulate would increase observable changes in intracellular zinc following supplementation. If validated, the mechanism described above would reveal the co-evolution of an *M. tb*-induced macrophage zinc response that parallels the induction of the *M. tb* divalent cation transporter CtpC [55]. In that model, the shared substrates of CtpC and ZIP8 including zinc and/or manganese [69, 364] would be transported both out of the bacteria and out of the phagosome. However, in the presence of CtpC, elevated zinc levels are not highly toxic to *M. tb* [55]. Furthermore, within the limitation of our assays to date,
any beneficial impact of ZIP8 on *M. tb* growth *in vitro* is limited to trends in zinc-supplemented macrophages and not statistically significant *in vivo*. Coordination of zinc efflux through the paired induction of CtpC and ZIP8 by *M. tb* may generate a redundant safeguard against zinc poisoning. In that scenario determination of the potential impact of ZIP8 on growth may require generation and infection with a *ctpC*-null mutant as previously described [55].

It should be considered that antibacterial activity of zinc against unadulterated *M. tb* has not been observed. All microbes require zinc for survival [216] and it enhances mycobacterial growth [348]. Zinc sequestration away from intracellular fungal pathogens is a validated host defense mechanism in macrophages [88, 217]. Furthermore the ancillary antioxidant properties of zinc afford protection of vulnerable sulfhydryl groups and damage by ROS [19] akin to those generated by high phagosomal concentration of iron [211] or copper [266]. Thus, high phagosomal zinc concentration may actually be protective for *M. tb*.

The transporter responsible for increased zinc concentration in the *M. tb* phagosome has not been identified. Others have proposed that induction and phagosomal localization of the cytosolic export protein ZnT1 following *M. tb* infection could be responsible for this increase [55]. Although ZnT1 is highly expressed constitutively in human macrophages, this increase was not recapitulated in our studies (Fig 15A). NRAMP1 is a zinc transporter [46] located on the mycobacterial phagosome [208, 212] that is capable of bidirectional zinc/proton anti-port against substrate and proton gradients [207]. As such
NRAMP1 is a potential contributor to either increased phagosomal zinc concentration or deprivation depending upon the pH and zinc content of the phagosome. ZIP8 and NRAMP1 also share iron as a substrate, raising the possibility that if ZIP8 is present on the *M.tb* phagosome there may be a dynamic interplay between the two transporters for the regulation of iron and zinc. Further, iron and zinc within the phagosome may antagonize the transport of one another such as they do in the intestine [10]. ZIP8-dependent iron transport across the phagosomal membrane has the potential to contribute to the previously proposed models of phagosomal iron deprivation [185] involving other transporters such as NRAMP1 [167] or ferroportin-1 [205]. That efflux could counteracting host protective iron-dependent generation of intraphagosomal ROS [211] and potentially explain the host protective effect of ZIP8 knockout (Fig 20B). ZIP8 co-localization studies could be expanded to include additional transporters including ZnT1 and NRAMP1. Furthermore, ZIP8 activity is pH dependent and potentially drives bicarbonate flux [44], therefore studies of its localization to the phagosome should be paired with knockdown to determine its impact on intraphagosomal pH, which is critical to maintenance of the intracellular mycobacterial niche [241].

Based on our findings, overall zinc has a pro-inflammatory effect on macrophages. It induces NF-κB activity (Fig 6A) and expression of pro-inflammatory cytokines during LPS exposure (Fig 8 and 9). Yet ZIP8 can also decrease macrophage NF-κB activity under alternative circumstances (Fig 2D)
and 6C). During *M. tb* infection, ZIP8 increases TNFα and IL-1β expression in alveolar macrophages (Fig 19A) indicating that it activates NF-κB activity *in vivo* in some cell types. Studies in human macrophages evaluating the impact of zinc supplementation and ZIP8 knockdown on *M. tb* induced pro-inflammatory cytokine expression and release should be conducted. Those investigations could resolve the functional consequences of zinc metabolism on macrophage inflammation during extended *M. tb* infection.

Similar to zinc (Fig 12A), iron has also been shown to inhibit C/EBPβ [165] and IL-10 production in murine macrophages through the actions of NRAMP1 [166], which results in reduced intracellular growth of *S. typhimurium*. Trafficking of multiple trace elements through different divalent cation transporters may impact the same signaling pathway through parallel mechanisms of divalent cation flux. Input from other transporters such as NRAMP1 may in part account for the incomplete restoration of IL-10 secretion during ZIP8 knockdown (Fig 10A) and the marginal impact of ZIP8 on *M. tb* growth (Fig 18B and 18C). Determination of the mechanism(s) governing zinc-mediated inhibition of IL-10 release during *M. tb* infection should include additional variables including iron supplementation and multiple transporters including NRAMP1 and ZIP8. Evaluation of the impact of transporter knockdown on C/EBPβ activity, subsequent IL-10 release and bacterial growth in the context of supplementation during *M. tb* infection in the MDM model are important follow up studies. They
could help to bridge the current findings and determine in what capacity ZIP8 is detrimental or beneficial to the host.

Cumulatively the preceding work supports a model of a host protective effect of zinc in macrophages. We believe that protection is achieved through inhibition of C/EBPβ activity (Fig 12A) and subsequent IL-10 release (Fig 17A) that reduces *M. tb* growth (Fig 17B) independent of ZIP8 induction (Fig 18B and 18C). Our studies also support a separate role for ZIP8 as a host susceptibility factor during *M. tb* infection (Fig 20B). The *in vivo* observations of a ZIP8-dependent increase in *M. tb* growth should be validated by additional studies. Additionally, it is possible that the impacts of zinc and ZIP8 on *M. tb* growth are countervailing and interfere with the determination of each-others growth effects. Characterization of the impact of physiological zinc and ZIP8-dependent zinc flux on *M. tb* growth could be achieved by evaluating pulmonary bacillary load after inducing subacute zinc deficiency and supplementation [160] with *M. tb* infection in the ZIP8 knockout murine model. Concurrent studies of C/EBPβ and/or NF-κB activation within isolated alveolar macrophages following infection should also be conducted in order to determine the functional significance of ZIP8-dependent zinc flux on transcriptional activation. Furthermore, in this experimental model *ex vivo* examination of intraphagosomal zinc content, ZIP8 phagosomal localization and its impact on lysosomal fusion could be conducted using fluorescent *M. tb* and a membrane permeable zinc fluorophore such as Zinpyr-1.
Additional experiments evaluating the impact of ZIP8 on long-term in vivo infection could also reveal host protective functions of ZIP8 in TB. ZIP8 increases IFN-γ expression by T cells [30]. That mechanism may be impacted by ZIP8 knockout in the myeloid specific ZIP8−/− murine model. IFN-γ is a major host protective cytokine responsible for activation of essential macrophage host defense function against M.tb [230]. IFN-γ is reduced in zinc deficiency [137] and induced by supplementation [140]. Elimination or reduction of IFN-γ levels in ZIP8 knockout mice could thereby reduce subsequent granuloma formation and control leading to increased dissemination and disease. Alternatively the observed trend in ZIP8-dependent host susceptibility to M.tb infection in vivo (Fig 19B) may also be the result of other ZIP8-dependent functions.

Observations that M.tb infection increases macrophage intracellular zinc accumulation leading to MTF-1 nuclear localization and transcriptional activity 24 hours after infection [55] are likely due, in part, to ZIP8-dependent zinc influx. ZIP8 is the only zinc transporter induced significantly by M.tb in macrophages at that time (Fig 15A). Similarly, in a separate murine Slc39a8 floxed;Col2a1-Cre chondrocyte-specific ZIP8 knockout model, ZIP8-dependent zinc increased intracellular zinc concentrations and MTF-1 dependent transcription of matrix metalloproteinases (MMPs) in during inflammation [72]. Subsequent MMP activity resulted in degradation of surrounding tissue. M.tb infection induces MMP production by macrophages and surrounding cells which results in pulmonary tissue destruction [365, 366]. In animal models, during the early phase of
mycobacterial granuloma formation, MMP release enhances macrophage recruitment to the site of infection [367], which is associated with increased macrophage infection and dissemination [226]. Furthermore MMP catalytic activity and function requires zinc [368]. The antibiotic doxycycline is the only known FDA approved MMP inhibitor and has been suggested as an adjunct antibiotic therapy because it reduces in vitro and in vivo M.tb growth [369]. In light of these findings it is intriguing to speculate that ZIP8 induction and subsequent zinc influx increases susceptibility to M.tb by driving MTF-1 transcription of MMPs resulting in tissue destruction, increased macrophage recruitment and bacterial growth. Initial experiments should test the impact of ZIP8-dependent zinc on MMP production and activity in vitro in M.tb-infected macrophages. Those experiments could be followed by evaluation of the impact of MMP inhibitors on ZIP8-dependent M.tb growth in ZIP8 knockout mice. MMPs contribute to granuloma destruction and tissue cavitation [365], which can enhance M.tb growth and dissemination. Thus, it is possible that ZIP8-dependent MMP production is a contributing factor in TB reactivation.

The realization that TB disease is more prevalent in populations with a higher risk for zinc deficiency (Fig 14) provides a broad translational justification for further investigation of human zinc metabolism during TB pathogenesis. The impact of zinc on host defense against infection is multifactorial and is based upon exploitation of the unique fundamental physiochemical properties of zinc. It is required for life and essential to both innate and adaptive immune function.
Zinc is necessary for maintenance of lymphocyte proliferation and function [136], which may be its principal benefit in host defense against TB since the eventual containment and control of the infection is a function of adaptive immunity [230]. However, manipulation of zinc distribution as a function of nutritional immunity within the vasculature and at a cellular level remains a vital yet understudied aspect of host defense.

The novel observation that *M. tb* dramatically impacts zinc metabolism in macrophages (Fig 15 and 16B), its targeted host cell, is potentially a critical aspect of disease pathogenesis. During infection, changes in zinc metabolism alter critical aspects of macrophage host defense including cytokine signaling (Fig 17A) and cellular zinc influx and distribution (Fig 16), which function in some ways to increase host defense functions of the infected cell. On the other hand, the prototypic, host-adapted intracellular pathogen, *M. tb*, appears to have evolved to exploit aspects of the host defense mechanisms employed by ZIP8 induction to its own benefit. This finding is not surprising when considering that *M. tb* has spent the entirety of the recorded history of humankind preying on the weaknesses of our physiological defenses. The studies herein further identify zinc as an armament of human health. This work advances our understanding of the body’s natural antimicrobial arsenal against *M. tb* and provides novel insight into the benefit of zinc, a widely available, inexpensive, immune modulatory micronutrient as a potential adjunct therapy for the prevention of TB.
BIBLIOGRAPHY


Google http://books.google.com/books?id=rh9rAAAAMAAJ

Google http://books.google.com/books?id=dCmFAAAAAIAAJ

HathiTrust Digital Library, Limited view (search only)
http://catalog.hathitrust.org/api/volumes/oclc/18683509.html

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SpringerLink http://dx.doi.org/10.1007/978-1-4471-3879-2

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gene expression by metal-regulatory transcription factor 1 (MTF1) in a model
37. Krezel A, Maret W. Dual nanomolar and picomolar Zn(II) binding
38. Krezel A, Maret W. Thionein/metallothionein control Zn(II) availability and
M. Nrf1 and Nrf2 play distinct roles in activation of antioxidant response element-
40. Maret W, Vallee BL. Thiolate ligands in metallothionein confer redox
41. Kimura T, Kambe T. The Functions of Metallothionein and ZIP and ZnT
42. Lichten LA, Cousins RJ. Mammalian zinc transporters: nutritional and
43. Leyva-Illades D, Chen P, Zogzas CE, Hutchens S, Mercado JM, Swaim
CD, et al. SLC30A10 is a cell surface-localized manganese efflux transporter,
and parkinsonism-causing mutations block its intracellular trafficking and efflux
versus Zn2+ uptake by the ZIP8 HCO3--dependent symporter: kinetics,


64. Kambe T, Tsuji T, Hashimoto A, Itsumura N. The Physiological, Biochemical, and Molecular Roles of Zinc Transporters in Zinc Homeostasis and Metabolism. Physiol Rev. 2015;95(3):749-84.


71. Wang CY, Jenkitkasemwong S, Duarte S, Sparkman BK, Shawki A, Mackenzie B, et al. ZIP8 is an iron and zinc transporter whose cell-surface


246. Bean AG, Roach DR, Briscoe H, France MP, Korner H, Sedgwick JD, et al. Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol Mycobacterium tuberculosis


284. Schlesinger LS, Horwitz MA. Phagocytosis of Mycobacterium leprae by human monocyte-derived macrophages is mediated by complement receptors


