Chapter 6
Metal Sequestration: An Important Contribution of Antimicrobial Peptides to Nutritional Immunity

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Abstract First-row transition elements are essential for all forms of life. During infection invading microbes must obtain these nutrients from their host. Vertebrates take advantage of this fact to combat invaders by sequestering essential nutrients, a defense known as nutritional immunity. The most well-characterized aspect of this defense is the iron-withholding response. Advances in elemental imaging have revealed that zinc and manganese are also sequestered during infection. The importance of nutritional immunity to host defense is emphasized by the increased susceptibility to infection when levels of these metals are elevated. This chapter will discuss iron, zinc, and manganese availability during infection, the impact of withholding these metals from invading pathogens, and the antimicrobial peptides utilized by the host to restrict the availability of these essential nutrients.

6.1 Introduction

Transition elements such as iron (Fe), zinc (Zn), and manganese (Mn) are essential for all forms of life. This essentiality stems from their numerous structural and catalytic roles. The importance of these metals to life is further emphasized by analyses of protein databases, which suggest that 30 % of all proteins utilize a metal cofactor (Andreini et al. 2008). Fe is highly versatile and is utilized by bacteria in numerous ways including in mononuclear enzymes, such as ribonucleotide reductase and superoxide dismutase; in Fe-S-containing enzymes, such as succinate dehydrogenase; and in heme-containing enzymes, such as cytochrome oxidase (Andreini et al. 2008; Py and Barras 2010; Mayfield et al. 2011). In bacteria, 4–6 % of all proteins

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are predicted to utilize Zn either catalytically, as in alcohol dehydrogenases, lyases, hydrolases, and Cu/Zn superoxide dismutases, or structurally, as in the Fur family of transcriptional regulators (Andreini et al. 2006; Vallee and Auld 1990; Waldron and Robinson 2009). Mn contributes to numerous processes and can serve as a cofactor for glycolytic enzymes, such as phosphoglycerate mutase and pyruvate kinase; signaling proteins, such as ppGpp synthetases; and superoxide dismutases (Kehres and Maguire 2003; Papp-Wallace and Maguire 2006).

During infection bacteria must acquire all of their nutrients from the host. Vertebrates take advantage of this fact by restricting the availability of essential transition elements. This host defense, known as nutritional immunity, is critical for defending against numerous pathogens (Hood and Skaar 2012; Weinberg 1974, 2009). The most well-characterized aspect of nutritional immunity is Fe restriction, but it has become apparent that the host also limits access to Zn and Mn to combat invaders (Kehl-Fie and Skaar 2010; Hood and Skaar 2012). This chapter will discuss the availability of essential transition metals during infection, the impact of withholding these metals on invading pathogens, and the host proteins produced by immune cells, which are utilized to restrict access to these essential nutrients during infection.

6.2 Nutrient Metal Availability During Infection

6.2.1 Iron Availability

While Fe is the most abundant transition element on earth, in vertebrates, free Fe is effectively nonexistent. The scarcity of Fe is driven by several factors (Cassat and Skaar 2013). First, Fe is highly compartmentalized, with the vast majority of this metal located in red blood cells in the form of heme bound to hemoglobin. Second, any released Fe is rapidly bound by transferrin. In response to infection, Fe availability is further restricted by the host. This further reduction in availability is driven by reduced intestinal Fe absorption, increased production of transferrin, and release of lactoferrin from neutrophil granules at sites of infection (Fig. 6.1). Additionally, phagocytes express natural resistance-associated macrophage protein 1 (NRAMP1), which removes Fe and Mn from the phagolysosome (Papp-Wallace and Maguire 2006). The importance of restricting Fe is seen in the increased ability of pathogens to replicate in Fe overloaded tissues and the increased susceptibility to infection of people suffering from Fe overload, whether due to environmental or genetic factors (Weinberg 1974, 2009).

In an attempt to obtain Fe during infection, pathogens express a myriad of different importers (Wandersman and Delepelaire 2004; Cassat and Skaar 2013). While the specific repertoire varies between pathogens, almost all bacteria express some form of Fe uptake system, which contributes to virulence. These systems include Fe transporters as well as heme and hemoglobin uptake systems. These latter systems allow bacteria to acquire Fe following the lysis of red blood cells. To counter these systems, extracellular hemoglobin and heme are rapidly scavenged by haptoglobin

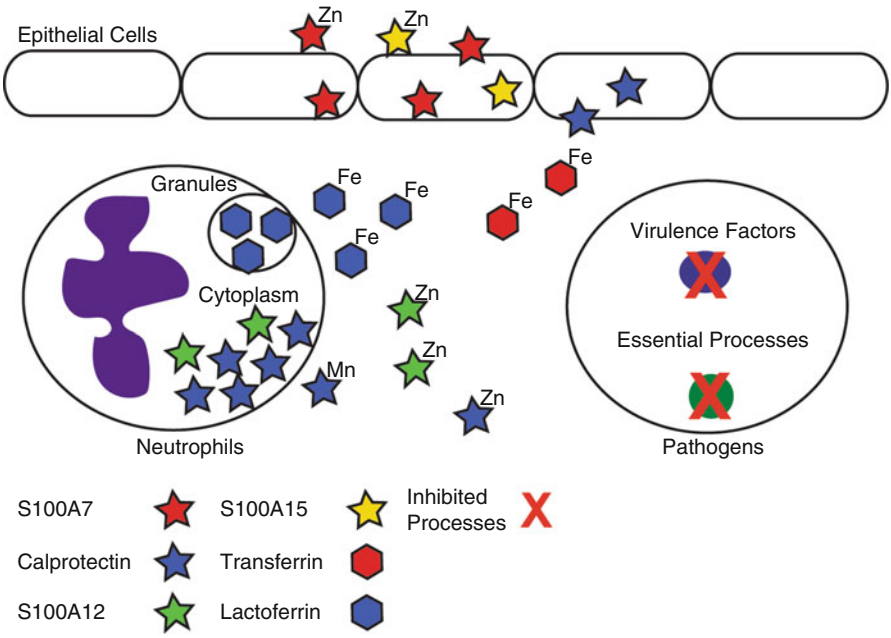

Fig. 6.1 Antimicrobial peptides sequester iron, zinc, and manganese during infection

and hemopexin, respectively. Bacteria also express receptors that allow them to utilize host Fe scavenging proteins including transferrin, haptoglobin, and hemopexin directly as nutrient sources. To acquire Fe, bacteria also produce siderophores, which have a higher affinity for Fe than transferrin. More recently it has become apparent that the host produces proteins that bind bacterial siderophores, such as lipocalin-2, preventing them from facilitating bacterial Fe acquisition. Lipocalin-2 is capable of binding Enterobactin and other similar siderophores (Goetz et al. 2002). An eight-stranded beta barrel protein, lipocalin-2, binds siderophores with subnanomolar affinity using a highly specific binding site comprised of lysine and arginine residues that interact tightly with the negatively charged siderophores (Goetz et al. 2002). To counter this defense, pathogens express "stealth siderophores," which cannot be bound by lipocalin-2 (Abergel et al. 2006; Raffatellu et al. 2009). The numerous strategies employed by host and pathogen reinforce the impact that wining the struggle for nutrient metals can have on the outcome of infection.

6.2.2 Zinc Availability

While Fe sequestration is the most well-characterized nutrient-withholding response, it has become apparent that Zn and Mn (discussed below) are also withheld from invading pathogens (Corbin et al. 2008; Kehl-Fie et al. 2013). However,

unlike Fe whose availability is always restricted, Zn and Mn appear to be specifically limited in response to infection. This realization has been largely driven by the application of advanced elemental imaging technologies, such as laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), to the study of infection (Corbin et al. 2008; Kehl-Fie et al. 2013). LA-ICP-MS produces a two-dimensional image of metal distribution within a tissue. The prototypic example of Zn restriction is the staphylococcal abscess (Corbin et al. 2008; Kehl-Fie et al. 2013). Utilization of LA-ICP-MS to investigate metal distribution during *S. aureus* infection revealed that the staphylococcal abscess is rendered virtually devoid of Zn, while the surrounding healthy tissue is metal replete. While the abscess is Zn deplete, total Zn levels in the organ remain unchanged (Kehl-Fie et al. 2013). Currently, the host factors responsible for sequestering Zn during infection are unknown. However, potential candidates include a subset of the S100 family of proteins (discussed below), such as calprotectin (CP), a heterodimer of S100A8 and S100A9 (also known as calgranulin A and B, Mrp8 and Mrp14, L1, and the CF-antigen), S100A12 (calgranulin C), and S100A7 (psoriasin), which are capable of binding Zn with high affinity (Kehl-Fie et al. 2011; Brophy et al. 2012; Moroz et al. 2011) (Fig. 6.1). While the specific expression profile of these proteins varies, they can be produced by epithelial cells and neutrophils. Furthermore, they are frequently found at high concentrations at sites of infection and can inhibit bacterial growth by sequestering Zn (Kehl-Fie and Skaar 2010; Lee and Eckert 2007; Gläser et al. 2005). Supporting the idea that the host is a Zn-limited environment, loss of Zn uptake systems in *Campylobacter jejuni*, *Salmonella enterica*, *Haemophilus ducreyi*, uropathogenic *E. coli*, *Brucella abortus*, *Streptococcus pyogenes*, *Acinetobacter baumannii*, and *Yersinia pestis* results in reduced bacterial virulence (Davis et al. 2009; Ammendola et al. 2007; Lewis et al. 1999; Sabri et al. 2009; Kim et al. 2004; Weston et al. 2009; Campoy et al. 2002; Hood et al. 2012; Bobrov et al. 2014). Notably, in an oral gastric model of *Salmonella* typhimurium infection, Zn sequestration appears to benefit the pathogen due to the expression of Zn importers, which allow *Salmonella* to outcompete the microbiota for Zn (Liu et al. 2012).

6.2.3 Manganese Availability

The observation that NRAMP1 removes Mn from the phagolysosome and contributes to controlling infection provided some of the first evidence that restricting Mn availability contributes to host defense (Papp-Wallace and Maguire 2006). The use of LA-ICP-MS has significantly expanded our knowledge regarding the scope of Mn sequestration during infection. Similar to Zn, LA-ICP-MS revealed that the staphylococcal abscess, but not the surrounding tissue, is rendered devoid of Mn (Corbin et al. 2008; Kehl-Fie et al. 2013). The localized Mn sequestration does not appear to impact total Mn levels in the organ (Kehl-Fie et al. 2013). Further investigation revealed that calprotectin (CP) contributes to this restriction, as CP-deficient mice do not remove Mn from staphylococcal liver abscesses (Corbin et al. 2008)

(Fig. 6.1). In addition to binding Zn, CP also binds Mn tightly, and the ability to sequester this metal contributes to the antimicrobial activity of the protein (Damo et al. 2013; Kehl-Fie et al. 2011). Mice that lack CP are more susceptible to bacterial and fungal pathogens, including *S. aureus*, *A. baumannii*, *Klebsiella pneumoniae,* and *Candida albicans* (Corbin et al. 2008; Kehl-Fie et al. 2013; Hood et al. 2012; Achouiti et al. 2012). CP is not the only host protein that contributes to Mn sequestration, as CP-deficient mice still remove Mn from staphylococcal kidney abscesses (Kehl-Fie et al. 2013). Similar to Fe and Zn, loss of Mn uptake systems in *S.* typhimurium, *B. abortus*, *Y. pestis*, *S. aureus*, *Streptococcus pneumoniae,* and *S. pyogenes* results in reduced virulence (Dintilhac et al. 1997; Berry and Paton 1996; Anderson et al. 2009; Bearden and Perry 1999; Horsburgh et al. 2002; Janulczyk et al. 2003; Sun et al. 2009; Kehl-Fie et al. 2013; Janakiraman and Slauch 2000). The importance of sequestering Mn is highlighted by the observation that a staphylococcal strain lacking Mn importers has a virulence defect in wild-type mice but is able to grow as well as the parental strain in the livers of CP-deficient mice (Kehl-Fie et al. 2013).

6.3 The Impact of Metal Limitation

Host-imposed metal limitation can impact the host-pathogen interaction in two ways. First, metal limitation causes directed changes in gene expression by invaders, which are intended to minimize the impact of nutrient limitation. Second, metal starvation can inactivate bacterial metalloenzymes disrupting essential metabolic pathways and processes. It should be noted that while the impact of metal starvation is likely to have some common aspects, the specific response and processes disrupted are likely to be as diverse as the pathogens that infect the human host. The directed bacterial response is frequently controlled by metal-responsive transcription factors, such as Fur (Fe), Zur (Zn), and MntR (Mn), which directly sense intracellular metal availability (Lee and Helmann 2007). Generally, these regulators repress expression when their cognate metal is available but not when it is limiting. These transcription factors frequently regulate the expression of metal uptake systems and metal-independent isozymes. They can also activate metal-sparing responses, as observed by Fur regulation of the small RNA RhyB in *E. coli*. Expression of RhyB leads to reduced expression of nonessential Fe-dependent processes increasing Fe availability for essential Fe-dependent processes (Lee and Helmann 2007). Metal limitation often indicates interaction with a host, and in some bacteria, metal-responsive regulators also regulate the expression of virulence factors (Troxell and Hassan 2013).

In addition to the directed changes, host-imposed metal limitation can also inactivate metal-dependent processes. While the specific processes that are inhibited by nutritional immunity are largely unknown, the increased susceptibility to infection when metals are not sequestered indicates that pathogens experience metal starvation during infection. This idea is supported by studies using wild-type and

CP-deficient mice, which found that host-imposed Mn starvation inhibits Mn-dependent staphylococcal superoxide dismutase activity during infection (Damo et al. 2013; Kehl-Fie et al. 2011). The inhibition of SOD activity in turn renders *S. aureus* more sensitive to neutrophil-mediated killing (Kehl-Fie et al. 2011). These results indicate that host-imposed metal starvation not only reduces bacterial growth but can also enhance the efficacy of the immune response by inhibiting virulence factors. While the impact of nutritional immunity on invading pathogens is still being elucidated, it is clear that withholding Fe, Zn, and Mn is a critical weapon utilized by the host.

6.4 Antimicrobial Peptides Involved in Metal Sequestration

6.4.1 Lactoferrin

Lactoferrin is a member of the transferrin family of Fe-binding proteins. Transferrin transports Fe within blood and other bodily secretions and contributes to the constitutive restriction of this metal. Lactoferrin is present within the granules of polymorphonuclear leukocytes where it acts as a crucial component of the innate immune response to infection (Orsi 2004). Additionally, lactoferrin can be found in breast milk, tears, and saliva. Similar to other members of the transferrin family, lactoferrin is an ~80 kDa protein comprised of two domains, an N-terminal domain (N lobe) and a C-terminal domain (C lobe). In lactoferrin, the two lobes are connected via a short helical stretch. This differs from transferrin where this linker is unstructured. The N lobe and C lobe share ~40 % sequence identity with each other resulting in two homologous halves (Wally and Buchanan 2007). Each lobe is comprised of two alpha/beta fold subdomains with the metal-binding site located at a deep pocket between the subdomains. Hence, each lactoferrin molecule can bind two Fe atoms. The ligands responsible for metal chelation are conserved throughout the transferrin family and in lactoferrin are Y92, Y192, D60, and H253 (Mizutani et al. 2012). Additionally, two oxygen ligands are provided by CO_3^{2-}, which is synergistically bound with Fe. Fe binding is tightly regulated and coordinated through conformational changes in lactoferrin (Baker and Baker 2004). In the apo state, lactoferrin forms an open structure, with the alpha/beta subdomains rotated as rigid bodies between 20° and 50° away from each other (Anderson et al. 1990). Upon Fe binding, the alpha/beta subdomains of each lobe close forming a much more compact structure. These conformational dynamics are critical for the controlled binding and release of Fe. It is suggested that CO_3^{2-} ion binds first to the open conformation (Baker and Baker 2004). This creates a four coordinate Fe-binding site together with the two tyrosines (Khan et al. 2001). Once Fe is bound, conformational sampling will select for the closed state and allow D60 and H253 to act as ligands and complete the full coordination site. This mechanism allows for the high-affinity ($K_d \sim 10^{-20}$ M) hexadentate coordination of Fe, while also permitting reversibility (Baker and Baker 2004).

6.4.2 S100 Proteins

The S100 subclass of EF hand family of calcium (Ca)-binding proteins are small, acidic, alpha helical proteins that are found in vertebrates. Their expression is tissue specific, and their regulation has been correlated with a wide range of diseases including autoimmune disorders, cancer, and neurological disorders (Heizmann et al. 2002). S100 proteins are approximately 100 amino acids long and typically form homodimers arranged in an antiparallel fashion. Each subunit contains two EF hand Ca-binding motifs connected by a hinge region.

The first crystal structure of a Zn-bound S100 protein was determined for S100A7 (Brodersen et al. 1999). This structure revealed the presence of two identical Zn-binding sites located at opposite ends of the dimer interface containing three histidines and one aspartic acid. In the S100A7 dimer, each binding site is comprised of H86 and H90, arranged in an HXXXH motif from one subunit and H17 and D24 from the other. These binding sites are distinct from the Ca-binding sites, which are characteristic of S100 proteins. While the binding sites are distinct, the presence of either Ca or a transition metal increases the affinity for the other (Brophy et al. 2012; Hayden et al. 2013; Moroz et al. 2011). This three His Asp site is the canonical transition metal-binding site in S100 proteins. Zn-bound structures have also been determined for S100B, S100A12, and S100A15 and suggest that the canonical binding site is largely conserved; however, there is some variation (Murray et al. 2012; Ostendorp et al. 2011; Moroz et al. 2009, 2011). For example, while S100A15 shares 93 % sequence identity with S100A7, originally it was thought to not bind Zn, as the conserved aspartate in the canonical binding site has been replaced by glycine. However, the crystal structure of Zn-bound S100A15 reveals a unique binding motif where a chloride ion acts as the fourth ligand, replacing the conserved aspartate (Murray et al. 2012).

Of the S100 proteins, the metal-binding properties of CP have received considerable attention. This interest is due to the demonstrated contribution of CP to host defense and the sequestration of Mn during infection (Corbin et al. 2008; Kehl-Fie et al. 2013; Hood et al. 2012; Achouiti et al. 2012). CP comprises ~50 % of the total protein in the neutrophil cytoplasm and can be expressed by epithelial cells when induced by proinflammatory cytokines such as IL-22 (Blaschitz and Raffatellu 2010; Gebhardt et al. 2006). At sites of infection, CP concentration can be in excess of 1 mg/ml (Clohessy and Golden 1995). CP is capable of binding two Zn ions with picomolar affinity (K_d) but only one Mn ion with nanomolar or subnanomolar affinity (Kehl-Fie et al. 2011; Brophy et al. 2012). Consistent with CP imposing metal starvation, the addition of excess Mn and Zn or mutation of both transition metal-binding sites eliminates the antimicrobial activity of CP (Corbin et al. 2008; Kehl-Fie et al. 2011).

Due to the heterodimeric nature of CP, this protein has two nonidentical transition metal-binding sites. The first site (S1) was originally predicted to be comprised of residues H17 and H27 from S100A8 and H91 and H95 from S100A9, with a fourth histidine replacing the aspartate found in the canonical S100 transition metal-binding site (Kehl-Fie et al. 2011; Korndorfer et al. 2007). However, subsequent

investigations revealed that two additional histidines, H103 and H105 from S100A9, also contribute to metal binding (Damo et al. 2013). The second site (S2) is comprised of H83 and H87 of S100A8 and H20 and D30 from S100A9 and is effectively identical to the canonical site found in other S100 proteins (Kehl-Fie et al. 2011; Korndorfer et al. 2007). Site 1 binds both Mn and Zn tightly, while S2 only binds Zn tightly (Damo et al. 2013; Brophy et al. 2013). A high-resolution crystal structure of Mn-bound CP revealed that H103 and H105 from a C-terminal extension of S100A9 also contribute to Mn binding (Damo et al. 2013). The six histidines in S1 form an almost perfect octahedral coordination site, with the C-terminal extension wrapping around the Mn ion. Subsequent solution studies confirmed the near-perfect octahedral geometry and the importance of H103 and H105 to Mn binding (Brophy et al. 2013). While loss of the two histidines disrupts the ability of S1 to bind Mn tightly, it appears to have negligible impact on Zn binding (Damo et al. 2013). The C-terminal extension is unique to S100A9 among the S100 proteins and provides an explanation for why none of the other S100 proteins assayed to date bind Mn (Damo et al. 2013; Brophy et al. 2013). Similar to other S100 proteins, there appears to be communication between the EF hands and the transition metal-binding sites, as the presence of Ca increases the affinity of CP for Zn and Mn. In the absence of Ca, CP has negligible affinity for Mn, and Zn binding is substantially weaker (Brophy et al. 2012; Hayden et al. 2013). This altered affinity has been proposed to serve as a switch, allowing CP to accumulate to high levels in the Ca-poor cytosol of neutrophils without negatively impacting the cell but bind Mn and Zn tightly when released into the Ca-rich extracellular space.

6.5 Conclusions

As invading pathogens must obtain essential nutrients from their host, nutritional immunity is a powerful host defense mechanism. While canonically associated with the Fe-withholding response, the application of advanced elemental analysis has revealed that other essential metals, such as Mn and Zn, are also withheld from invaders. The expansion of nutritional immunity to include Mn and Zn leads to more questions. What are the host factors that restrict the availability of these metals during infection? What are the bacterial processes that are disrupted by nutritional immunity? How do successful pathogens overcome this host defense, allowing them to cause disease? Answering these questions will aid in the development of therapeutics that are intended to augment the efficacy of nutritional immunity.

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