Nutritional Immunity: S100 Proteins at the Host-Pathogen Interface

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The S100 family of EF-hand calcium (Ca2+)-binding proteins is essential for a wide range of cellular functions. During infection, certain S100 proteins act as damage-associated molecular patterns (DAMPs) and interact with pattern recognition receptors to modulate inflammatory responses. In addition, these inflammatory S100 proteins have potent antimicrobial properties and are essential components of the immune response to invading pathogens. In this review, we focus on S100 proteins that exhibit antimicrobial properties through the process of metal limitation, termed nutritional immunity, and discuss several recent advances in our understanding of S100 protein-mediated metal sequestration at the site of infection.

S100 proteins are EF-hand Ca2+-binding proteins involved in a diverse array of both intracellular and extracellular regulatory functions (1–6). Over 20 S100 proteins have been identified, and all have a characteristic dimeric structure distinct from other EF-hand proteins (7). Like many EF-hand proteins, Ca2+ signaling function is associated with a binding-induced conformational change exposing a hydrophobic patch that generates specificity for target proteins (8, 9). Within the cell, S100 proteins regulate numerous important processes including Ca2+ homeostasis, energy metabolism, and cell proliferation and differentiation. Remarkably, certain S100 proteins can be secreted and/or released by cells, and among these, some play an important role during infection and inflammation (1). In particular, extracellular S100 proteins can act as damage-associated molecular pattern (DAMP)3 proteins and initiate a pro-inflammatory immune response through interaction with pattern recognition receptors and the receptor for advanced glycation end products (RAGE) (10, 11). Furthermore, through the process of nutrient metal limitation, several S100 proteins have been shown to be antimicrobial and play a key role in host defense at the host-pathogen interface (12–17). In this review, we provide insight into structure and function of the three S100 proteins with antimicrobial and inflammatory properties: S100A7 (psoriasin); S100A8/S100A9 (calprotectin; calgranulin A and B; MRP-8 and 9); and S100A12 (calgranulin C).

Key Properties of S100 Proteins with Antimicrobial Activity

Structure and Metal Binding

The basic unit of EF-hand proteins is a helix-Ca2+-binding loop-helix motif; these motifs are typically packed in pairs to form a stable globular four-helix bundle domain (8). Each S100 protein contains a distinctive S100-specific N-terminal EF-hand motif and a C-terminal canonical EF-hand motif (Fig. 1). The fundamental structural unit of S100 proteins is a highly integrated antiparallel dimer (7); all S100 proteins form this structure as homodimers, and some will also heterodimerize. S100A8 and S100A9 are unique among all members of the family because they preferentially form a heterodimer (18), which is termed calprotectin based on its role in innate immunity. S100 proteins are also known to form higher order oligomers, usually mediated by high levels of Ca2+ or Zn2+.

Like other EF proteins that function in signaling, the binding of Ca2+ causes a conformational change, in this case within each S100 protein subunit (Fig. 1A) (8, 9, 19). Ca2+ plays an important role in the functional duality of calprotectin. Inside cells, where the basal level of Ca2+ is in the nanomolar range, calprotectin can serve as a sensor of Ca2+ signals, which are associated with ~100-fold increase in Ca2+ concentration into the micromolar range. This in turn results in the binding of ions, conformational change, and interaction with intracellular target proteins. Ca2+ is also known to stimulate formation of higher order oligomers of S100 proteins, including S100A8/S100A9 tetramers that have been suggested to play a role in some of calprotectin’s activities (15, 20–22). In the extracellular milieu, S100 proteins do not function as Ca2+ sensors because Ca2+ concentration is in the mM range and the proteins are perpetually Ca2+-bound. Secretion of S100 proteins therefore causes a change in the functional properties. Thus, it has been proposed that Ca2+ may act as the molecular switch between the intracellular and extracellular functions of calprotectin (21). Because extracellular calcium is constant, the molecular switch can also be viewed as the secretion of the protein. Regardless, extracellular function of calprotectin is governed by being Ca2+-bound. Importantly, Ca2+ has been shown to stimulate the binding of transition metals in calprotectin, which as discussed below is essential for its role in host defense against pathogens (2).

S100 proteins are distinguished from other EF-hand proteins by the presence of two transition metal binding sites at the dimer interface. In calprotectin, the first transition metal binding site (Site I) is capable of binding both zinc (Zn2+) and manganese (Mn2+) with high affinity (Kd (Zn2+) ~10−9 M, Kd (Mn2+)}
Expression and Regulation of S100 Proteins

To maximize the protective function of antimicrobial S100 proteins while simultaneously maintaining immune system homeostasis, expression of S100 proteins is tightly regulated (1, 4). Calprotectin and S100A12 are primarily expressed in cells of myeloid origin, such as neutrophils, monocytes, and early macrophages (5, 26, 27). In neutrophils, calprotectin accounts for over 40% of the cytoplasmic fraction, highlighting its importance in the neutrophilic immune response (28, 29). Expression of calprotectin and S100A12 can be induced in keratinocytes, endothelial cells, and epithelial cells during inflammation (27, 30–34). Furthermore, various in vitro studies have shown that induction of macrophages with pro-inflammatory cytokines can lead to the expression and release of calprotectin and S100A12 (35–37). Secretion of S100 proteins including calprotectin and S100A12 is facilitated by: (a) active release through intact microtubule networks in a Golgi-independent pathway (38); (b) release during the formation of neutrophil extracellular traps (39); or (c) release through passive release during cell necrosis (40). At some sites of infection and inflammation, calprotectin concentrations exceed 1 mg/ml, suggesting massive expression and/or mobilization of this protein during infection (41). S100A7 is constitutively expressed in skin at relatively high levels, and expression is amplified in keratinocytes upon induction by pro-inflammatory cytokines IL-17 and IL-22 and bacterial products, such as flagellin (42). The differential expression profiles of S100 proteins allow for an immediate antimicrobial response upon infection in certain tissues, while limiting potentially detrimental inflammatory responses associated with each of these proteins. The capacity for S100 proteins to mediate inflammation and the potential link to chronic inflammatory disease will be discussed below.

S100 Proteins during Infection

Inflammatory Response and Regulation

In the extracellular matrix, S100 proteins can act as potent modulators of inflammation. Once released by cells, these proteins are classified as DAMPs because of their important role in regulating inflammatory responses (10). Extracellular S100 proteins can exhibit chemokine- and cytokine-like activity, initiate pro- and anti-inflammatory responses, and interact with pattern recognition receptors. Growing evidence suggests that S100-mediated inflammation is driven by endogenous interaction with pattern recognition receptors including RAGE and Toll-like receptors (10, 11). It has been demonstrated that calprotectin is an endogenous agonist of TLR4 (43). Binding to TLR4 and several other components of the lipopolysaccharide complex initiates a signaling cascade that promotes inflammation, autoimmunity, and tumor development in an NF-κB-dependent manner (43–45). Apart from TLR4, evidence also suggests that calprotectin, S100A7, and S100A12 each independently interact with RAGE. S100 protein activation of RAGE drives an NF-κB-mediated pro-inflammatory response and recruitment of neutrophils, monocytes, and macrophages (11, 46–48). Additionally, calprotectin has been suggested to activate pro-inflammatory cytokine production in monocytes and macrophages through NF-κB and p38 MAPK pathways (47,
During infection, calprotectin also acts as a chemotactic for neutrophils and can promote neutrophil adhesion at the site of infection in a RAGE-independent manner (50). Beyond their role in the pro-inflammatory response during infection, these three S100 proteins may also have important anti-inflammatory functions. For example, calprotectin appears to have the ability to scavenge reactive oxygen species (ROS) (51, 52). It has been proposed that scavenging enables calprotectin to minimize collateral damage associated with neutrophil ROS (27). Furthermore, calprotectin inhibits growth or promotes apoptotic and autophagy-like death in several cell types including macrophages, lymphocytes, endothelial cells, and tumor cells (53, 54). Taken together, these data suggest that the impact of the three S100 proteins on the immune response is complex and likely dependent on a combination of factors including the local concentration of metal ions, the distribution of immune cells, and the site of infection.

An important concept associated with S100 proteins is the regulatory role that metal binding plays in modulating structure and functions of these proteins. As noted above, Zn^{2+} and Ca^{2+} stimulate oligomerization of S100A12. Interestingly, oligomerization is believed to be required for RAGE binding and subsequent inflammation (55). Evidence also suggests that the RAGE-dependent chemo-attractant activities of S100A7 may be dependent on Zn^{2+} binding (56). Similarly, Ca^{2+} binding stimulates transition metal binding, which is essential for extracellular functions. Binding of Zn^{2+} has also been suggested to mediate calprotectin’s apoptosis-inducing activity (57). The ability to control function via concentration or localization of transition metal ions may allow for the immense adaptability and diversity of activities seen in many S100 proteins.

### S100 Proteins Associated with Inflammatory Diseases

Table 1 lists the specific association of S100A7, S100A8/S100A9, and S100A12 with inflammatory diseases (Table 1), which is not surprising given their expression patterns and immunomodulatory effects during inflammation. Calprotectin and S100A12 are present at high concentrations in inflamed tissues that harbor neutrophil and monocyte infiltrate. Similarly, S100A7 is present at high abundance in inflamed skin. It has been suggested that release of these S100 proteins during inflammation is at least partially associated with Ca^{2+} influx upon activation of monocytes (35). Calprotectin and S100A12 have been associated with various inflammatory diseases such as rheumatoid arthritis, psoriasis, and inflammatory bowel disease (10, 32, 58). Furthermore, these proteins are believed to play an important role in several cancers (59–61). Similarly,
S100A7 is linked to several inflammatory skin diseases, including psoriasis and atopic dermatitis (42, 62). The mechanism by which the three antimicrobial S100 proteins mediate autoimmunological, inflammatory, and pro-cancer activities is directly associated with their role as potent immune modulatory DAMPs. Through the activation of pro-inflammatory signaling cascades at the sites of disease, these proteins contribute to a positive feedback loop that produces overly active inflammatory responses and pro-tumor microenvironments. Due to their association with inflammation, these proteins have also been exploited as noninvasive biomarkers for several disorders, including inflammatory bowel disease, rheumatoid arthritis, and colon cancer (63–65). In addition, they represent logical targets for therapeutics that aim to minimize aberrant inflammation associated with disease.

Hypercalprotectinemia is an extremely rare inflammatory disorder that is associated with extraordinarily high levels of calprotectin. The few patients who have been described with this disorder have abnormally high levels of Zn2+ in their tissues and develop aberrant systemic inflammatory responses (66–69). Chronic inflammation leads to symptoms such as dermal ulcers, folliculitis, and anemia. To this point, the mechanisms that drive this rare disease are largely unknown; however, it is postulated that calprotectin catabolism may be defective in these individuals (66). Furthermore, it is possible that calprotectin release into the extracellular matrix is dysregulated during hypercalprotectinemia, leading to increased release of calprotectin and a subsequent hyperactive systemic inflammatory response.

**Nutritional Immunity**

During infection, invading bacterial pathogens require access to essential transition metals to colonize the host and cause disease (70). To combat this, the host exploits the pathogen’s need for nutrient metals by producing factors that limit metal availability and starve pathogens in a process termed nutritional immunity. The S100 protein calprotectin is the best studied of these nutritional immunity factors. Calprotectin has broad-spectrum antimicrobial activity based on its ability to sequester Zn2+ and Mn2+ at the site of infection. Several recent studies have shown that it inhibits growth of numerous important human pathogens in vitro including *Staphylococcus aureus*, *Acinetobacter baumannii*, *Candida albicans*, and *Helicobacter pylori* (13, 14, 16, 39). In each case, calprotectin-mediated inhibition is reversed by ablating calprotectin metal binding activity through mutagenesis or by complementing with excess Zn2+ or Mn2+. Utilizing S100A9−/− mice, which are calprotectin-deficient, several studies have shown that calprotectin is important for protecting against bacterial infection in murine models of *A. baumannii* pneumonia, *H. pylori* gastric infection, *S. aureus* systemic infection, and *C. albicans* subcutaneous and pulmonary infections (12–14, 16, 39). These *in vivo* studies demonstrate the importance of calprotectin-mediated nutritional immunity during infection.

There have been few studies to establish the microbial processes that are impacted by calprotectin metal limitation. Interestingly, calprotectin-mediated sequestration of Mn2+ at the site of infection increases *S. aureus* susceptibility to neutrophil killing by superoxide through the inhibition of the staphylococcal Mn2+-dependent superoxide dismutase defense system. This is one example of how calprotectin-induced metal restriction impacts bacterial metabolism (16). These findings suggest that at the host-pathogen interface, calprotectin functions by outcompeting or stripping metals from pathogen metalloproteins, rendering them inactive and weakening their defenses to the host immune response. In some cases, pathogens have developed the ability to compete with calprotectin-mediated metal starvation in the gut. *Salmonella* serovar Typhimurium expresses a high affinity Zn2+ transporter (ZnuABC) to acquire Zn2+ during infection (17). This strategy confers resistance to calprotectin-mediated metal chelation and allows S. Typhimurium to thrive under conditions of inflammation in the gut and outcompete the resident commensal bacteria (17).

In addition to calprotectin, accumulating evidence supports a potential role for S100A7 in limiting bacterial infection through metal limitation (42, 72), although there are differences in the effect on different organisms. At low doses, purified S100A7 from keratinocytes has antimicrobial activity against *Escherichia coli*, but at higher concentrations, S100A7 exhibits killing activity against *Pseudomonas aeruginosa* and *S. aureus* (42). The antimicrobial activity of S100A7 is dependent on the limitation of Zn2+, as complementation with excess Zn2+ ablates bactericidal activity (42). Additionally, an alternative mechanism for antimicrobial activity has been proposed, by which S100A7 directly adheres to and reduces survival of *E. coli* and potentially other pathogens found on the epidermis (73). Through cross-linking to bacterial components, S100A7 may reduce survival and act as a physical barrier during infection. Further studies are needed to explore this phenomenon.

Human S100A12 possesses antimicrobial activity against several parasites (5, 72). However, it has been difficult to study in the context of infection because it is not present in mice. The mechanism of antimicrobial activity is unclear, but it is tempting to speculate that S100A12-mediated metal limitation plays a key role during infection. This hypothesis is supported by the antimicrobial properties observed with calcitermin, a protein homologous to the C terminus of S100A12. Calcitermin exhibits Zn2+-dependent antimicrobial activity against *P. aeruginosa*, *C. albicans*, and *E. coli* (74). It has also been suggested that Cu2+ bound to S100A12 actively produces superoxide, which could potentially be antiparasitic (72). Given the significant gaps in our knowledge regarding the role of S100 proteins in host defense against infection, it is clear that further research is needed to characterize whether S100A12 contributes to nutritional immunity. Furthermore, additional studies are required.
to define the mechanisms by which metal limitation impacts invading organisms. We believe that developing a greater understanding of the influence of metal limitation on an array of organisms will lay the groundwork for development of novel therapeutics to target critical pathogens.

Concluding Remarks and Future Challenges

Evidence strongly supports an important role for S100A7, calprotectin, and S100A12 as antimicrobial proteins that protect against infection (Fig. 2). However, these S100 proteins can also have a negative impact on the host by amplifying aberrant pro-inflammatory responses and potentiating disease (Fig. 2). It is clear that we are just beginning to understand the importance of S100 proteins and the roles they play in both inflammation and nutritional immunity. A detailed analysis of the mechanisms that regulate S100 protein function during infection and inflammation will improve our understanding of how immune homeostasis is maintained during health and disrupted during S100 protein-associated disease.

Critical insights have been obtained on the impact of S100 protein-mediated metal limitation at sites of infection. However, the impact of metal loading on S100 protein immunoregulatory effects at the site of infection remains largely unstudied. With the development of techniques such as CRISPR-CAS9 genome editing, we anticipate an accelerating pace of discovery. Interrogation of the metal binding sites of S100 proteins and performing in vivo characterization at the site of infection will provide critical new insights. It is anticipated that obtaining better understanding of these complex processes will allow for the development of therapeutics directed to S100 proteins and their targets during aberrant inflammatory disease states, while maintaining their essential role in nutritional immunity to invading pathogens.

Consideration must be given to the specific environment within the host where S100 proteins are interacting with the immune system and the pathogen. During infection, different tissues have dramatically altered metal concentrations, cell types, and stresses. S100 proteins may be involved in controlling transition metal distribution and homeostasis in infected tissue. Understanding how S100 proteins differentially function at diverse sites of infection and during infections by different organisms is essential for elucidating their role in nutritional immunity and inflammation. Key questions still remain on the pathogen side of the host-pathogen interface. Little is known about the targets of antimicrobial metal limitation during infection. Elucidation of these pathogen factors could lead to the development of novel drug targets that focus on the fundamental nutrient requirements of pathogens. Continued work studying S100 protein biology in these contexts is expected to lead to significant advances in our understanding of infection, autoimmunity, and cancer.

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MINIREVIEW: S100 Proteins at the Host-Pathogen Interface

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MINIREVIEW: S100 Proteins at the Host-Pathogen Interface

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MINIREVIEW: S100 Proteins at the Host-Pathogen Interface

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