THE EFFECTS OF CALPROTECTIN, A METAL BINDING PROTEIN, ON THE GROWTH OF BORRELIA BURGDORFERI.

by
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Abstract

*Borrelia burgdorferi*, the causative agent in Lyme disease, is unique in that it has evolved without the requirement for iron unlike other pathogens. Rather, *B. burgdorferi* accumulates other metals such as manganese and zinc. We find that calprotectin, a member of the S100 family of proteins known to bind manganese and zinc with two metal binding sites, is toxic to *B. burgdorferi*. *E. coli* grown in the same medium requires nearly ten times more calprotectin to show growth inhibition. This grown inhibition involves the zinc-binding site (site 2) of calprotectin as opposed to the other metal binding site (site 1), which binds manganese, copper, and zinc. We find that S100A12, another member of the S100 family of proteins known to bind zinc and copper, is also toxic to *B. burgdorferi* at doses similar to that of calprotectin. Metal analysis of *B. burgdorferi* yielded the surprising result that rather than withholding metals, calprotectin and S100A12 toxicity was associated with increases in manganese, copper, and zinc levels in *B. burgdorferi*. Furthermore, we find that calprotectin toxicity reflects direct binding of calprotectin to *B. burgdorferi* and this binding requires zinc-binding site 2. We provide a model in which calprotectin and perhaps other S100 proteins inhibit growth of *B. burgdorferi* not by starving pathogens of metals, but rather by causing cell toxicity by direct binding to the bacteria and inducing metal uptake.

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Introduction

Prevalence of Lyme disease

Lyme disease is a tick borne illness caused by the bacteria, *B. burgdorferi* (Steere, Coburn, & Glickstein, 2004). Lyme disease was named after a small town in Connecticut called Lyme. In 1975, there was a cluster of children in this town who were thought to have juvenile rheumatoid arthritis. Later, it became evident that the syndrome they had was a multisystem illness transmitted by a tick (Steere et al., 1977). Lyme disease is the most commonly reported vector borne illness in the United States. During the time between 1992 and 2013, the Center for Disease Control reported 430,450 confirmed cases in the United States. The disease incidence has risen from 11,603 confirmed cases in 1995 to 27,203 confirmed cases in 2013 (Fig. 1) (Adams et al., 2014; Bacon, Kugeler, Mead, Centers for Disease, & Prevention, 2008; Centers for Disease & Prevention, 2014). In the United States, the incidence of Lyme disease is highest in Northeastern, Mid Atlantic, and North Central regions. The following 14 states (Connecticut, Delaware, Maine, Massachusetts, Maryland, Minnesota, New Hampshire, New Jersey, New York, Pennsylvania, Rhode Island, Vermont, Virginia and Wisconsin) accounted for more than 96% of the confirmed cases for Lyme disease in 2014 (Fig. 2, 3) (Centers for Disease & Prevention, 2014).

Symptoms of Lyme disease vary by the three stages of the infection. Stage one is characterized by the localized rash called Erythema Migrans (EM). Stage two is disseminated infection followed by stage three, persistent infection (Asbrink, 1985). EM is a slowly expanding skin lesion caused by the localized reproduction of the bacteria
within the skin. EM can occur anytime between 3 to 14 days after the initial tick bite (Wormser et al., 2006). EM is also the most common feature, occurring in around 70-80% of cases (Steere & Sikand, 2003). There can also be other symptoms associated with EM such as fatigue, fever, headache, mild stiff neck, joint pain, and muscle pain (Steere, Bartenhagen, et al., 1983). If Lyme disease is not treated properly, it can spread to other parts of the body, which can lead to cardiac abnormalities, arthritis, and neurological defects such as facial palsy (Steere & Sikand, 2003). Some people continue to have symptoms such as musculoskeletal pain, neurocognitive difficulties, and fatigue that can last for years, which is termed chronic Lyme disease (Kalish et al., 2001).

Because the symptoms vary with stage, physicians must look into epidemiological cues and look into the potential exposure to infected ticks (Tugwell et al., 1997). Treatment includes doxycycline for early localized and disseminated infection. Children and pregnant women are not advised to take doxycycline but amoxicillin instead (Nichol et al., 1998). There are many factors in determining risk for Lyme disease such as human behaviors (Cromley, Cartter, Mrozinski, & Ertel, 1998), the density of tick population, feeding habits of ticks, and the animal hosts (Spielman, 1994). Preventative measures can include wearing protective clothing and repellants when walking around in wooded or grassy areas (Wormser et al., 2006).

*Borrelia burgdorferi*

*B. burgdorferi* is an obligate parasite, belonging to a family of spirochetes. In 1981, Burgdorfer and his team isolated the unrecognized spirochete from *Ixodes scapularis*
ticks (Burgdorfer et al., 1982). Afterwards, the spirochete was isolated from patients with Lyme disease in the United States (Steere, Grodzicki, et al., 1983). Spirochetes are helically shaped and motile. The outer cell membrane surrounds the protoplasmic cylinder complex, which consists of the cytoplasm, inner cell membrane, and peptidoglycan. They also have flagella that are located in the periplasmic space between the outer cell membrane and the protoplasmic cylinder (Fig. 4). Spirochetes can vary by length, diameter, and tightness of the coils (Barbour & Hayes, 1986). The genome of *B. burgdorferi* is small, consisting of a linear chromosome of 910, 725 base pairs and at least 17 linear and circular plasmids. The chromosome contains 853 genes, which encode a fundamental set of proteins necessary for DNA replication, transcription, translation, solute transport, and energy metabolism. It does not have any genes for the synthesis of amino acids, fatty acids, enzyme cofactors, and nucleotides. The small genome size and the absence of biosynthesis pathways can explain why *B. burgdorferi* is difficult to culture in vitro and requires serum-supplemented tissue culture medium (Fraser et al., 1997). The medium used to grow *B. burgdorferi* is called Barbour-Stoenner-Kelly (BSK) medium. It was initially developed by Richard Kelly in 1971, while Stoenner and Barbour made more modifications to the medium, resulting in BSK II medium. It contains over thirteen ingredients, including a mixture of vitamins, minerals, amino acids, nucleotides, Yeastolate, and Bovine Serum Fraction V, supplemented with rabbit serum. BSK II contains several metals, including iron (≈12 µM), copper (≈6 µM), zinc (≈3 µM) and very low levels of manganese (≤0.2 µM) (Table 1). The bacteria grow optimally at around 32° C. Overall, *B. burgdorferi* grows slowly, doubling every 12-18 hours in laboratory cultures (Barbour, 1984; Kelly, 1971; Stoenner, 1974).
Tick Biology

The principal vector of Lyme disease in eastern North America is the *Ixodes scapularis*, (or *I. dammini*) also known as the blacklegged tick or deer tick. These ticks are mostly abundant in areas in Northeastern, Mid-Atlantic, and North Central United States. *I. scapularis* nymphs feed mostly on white footed mice, leading to a efficient cycle of transmission. *I. scapularis* adult ticks prefer deer as its host. Deer are immune to *B. burgdorferi*, but they do play an important role in transporting and supporting tick population (J. Piesman & Gern, 2004). While *I. scapularis* can also be found in the Southeastern part of the United States, the ticks in this area are less infected. One of the reasons is that the ticks in this region feed primarily on lizards, and lizards are not susceptible to infection by *B. burgdorferi* (Oliver, Cummins, & Joiner, 1993).

In the western United States, *I. pacificus* is the primary vector, also called the western-blacklegged tick. *I. pacificus* is found along the Pacific Coast from British Columbia to Baja California Norte, Mexico (Kain, Sperling, & Lane, 1997). *I. pacificus* is usually not infected because they also mostly feed on lizards (Brown & Lane, 1992).

The three stages of a tick are larvae, nymph, and adult tick. Ticks have to stay attached for 24 hours or more for infection to occur (J. Piesman, Mather, Sinsky, & Spielman, 1987). The life cycle of *I. scapularis* lasts 2 years. During the months of August to September of year one, the larvae feed on mice, birds, and other small animals (Joseph Piesman & Spielman, 1979). During year two in May to July, the nymphs feed either on other small rodents or humans. These nymphs mount to adults that same fall. Adult ticks
usually feed on large animals such as deer or humans. The female adults lay eggs and the life cycle continues (Yuval & Spielman, 1990). Ticks are uninfected when they hatch, but acquire the spirochete by feeding on infected reservoir hosts including mice, shrews, small mammals and some birds. The infected ticks then transmit the spirochete to new reservoir hosts during subsequent feedings. Humans are dead end hosts (J. Piesman & Gern, 2004) (Fig. 5). While there are reports of potential transovarial transmission (TOT) by *B. burgdorferi* (Hamer, Tsao, Walker, & Hickling, 2010), there is also evidence that TOT by *B. burgdorferi* does not occur. Rather than *B. burgdorferi*, *B. miyamotoi*, which is antigenically and phylogenetically related to *B. burgdorferi*, is most likely transovarially transmitted (Rollend, Fish, & Childs, 2013).

*Metals in B. burgdorferi*

Metals, including iron, zinc, manganese and copper, serve as cofactors for nearly 40% of all enzymes in biology (Waldron, Rutherford, Ford, & Robinson, 2009). Therefore, all living organisms must acquire trace levels of these metals to survive. As part of the innate immune response in vertebrates, the hosts sequesters these transition metals in order to starve pathogens of nutrients, which is termed “nutritional immunity” (Weinberg, 1974).

In the past, studies on nutritional immunity have primarily focused on host sequestration of iron. Most organisms need high levels of iron as a co-factor for numerous metalloenzymes involved in energy generation, DNA replication, oxygen transport, and protection against oxidative stress. Bacterial pathogens typically need to acquire iron
within their vertebrate host to replicate and cause disease (Kehl-Fie & Skaar, 2010). The host takes advantage of this iron requirement of the pathogen by shutting down circulating iron through a condition known as anemia of inflammation. During infection, cytokines, such as interleukin (IL)-1, IL-6, IL-22 or bacterial LPS, induce the production of hepcidin, a master regulator in iron homeostasis, in the liver (Elizabeta Nemeth et al., 2002). Hepcidin binds to ferroportin, an iron export protein, leading to internalization and degradation of ferroportin, which then results in a decrease in cellular iron export from macrophages and intestinal cells (E. Nemeth et al., 2004) leading to a reduction of circulating iron levels (E. Nemeth et al., 2004a). At the onset of infection, the human host withholds even more iron by secreting proteins such as transferrin, lactoferrin and siderocalin into the blood stream (Flo et al., 2004; Goetz et al.; Singh, Parsek, Greenberg, & Welsh, 2002).

In order to colonize in the human host, bacteria must overcome this limitation of iron (Payne, 1993; Wooldridge & Williams, 1993). Bacteria compete with the host to acquire iron by high affinity iron uptake mechanisms including heme acquisition systems, transferrin/lactoferrin receptors, and siderophore-based systems (Ratcliff, 1988). However, such elaborate systems for iron acquisition do not seem to exist in B. burgdorferi.

Posey and Gherardini have shown that B. burgdorferi is unique in that it has evolved without the requirement for iron. There are no iron-requiring cytochromes, respiratory proteins, or tricarboxylic acid metalloenzymes detected in the purified inner membranes
or encoded within the genome. *B. burgdorferi* does not express any iron specific enzymes. Moreover, iron chelators did not affect the growth of *B. burgdorferi* or alter gene expression patterns. There also is no detectable accumulation of iron inside the cell. To date, *B. burgdorferi* is the only organism known to not accumulate nor require iron (Posey & Gherardini, 2000). This unique independence from iron makes *B. burgdorferi* quite resistant to the anemia of inflammation response of the host.

While it does not need iron, *B. burgdorferi* does accumulate other metals such as manganese and zinc. It has been shown that *B. burgdorferi* cells can accumulate unusually high levels of manganese that are 2 orders of magnitude higher than that of *E. coli* grown in BSK medium. Most iron-philic organisms such as *E. coli* and *Saccharomyces cerevisiae* have much higher levels of iron than manganese. The opposite is true for *B. burgdorferi*, with high manganese and virtually non-existent iron (Aguirre et al., 2013).

The only metal ion transporter characterized to date in *B. burgdorferi* is the Mn transporter BmtA (Ouyang, He, Oman, Yang, & Norgard, 2009). BmtA is related to the Zip family of zinc transporters (Guerinot, 2000) but shows no homology to manganese transporters of other bacteria including natural resistance-associated macrophage protein (Nramp) type Mn transporter, the ATP binding cassette (ABC) Mn permease, and the P-type ATPase Mn transporter (Archibald, 1986; Jakubovics & Jenkinson, 2001; Papp-Wallace & Maguire, 2006). BmtA is critical for the uptake of manganese in *B. burgdorferi* when grown in BSK II media, which has \( \leq 0.2 \ \mu M \) manganese and \( \approx 3 \ \mu M \)
µM zinc (Table 1). BmtA is also responsible for the extremely high manganese accumulated in this bacteria (Norgard et al., 2009, Aguirre et al., 2013). A strain of B. burgdorferi lacking BmtA did not have a substantial growth defect in culture, however, this mutant was completely avirulent in mice. It was also unable to infect and proliferate in ticks, showing that BmtA and high levels of manganese are crucial for B. burgdorferi’s infectious life cycle. In B. burgdorferi, manganese is needed to activate superoxide dismutase enzymes to guard against oxidative stress and to also regulate expression of the Borrelia Oxidative Stress Regulator (BosR) (Aguirre & Culotta, 2012; Bryan Troxell, Xu, & Yang, 2012; B. Troxell & Yang, 2013; B. Troxell et al., 2013).

Zinc is also an important metal for B. burgdorferi, however there are no known Zn transporters in this spirochete. Zinc is critical for activity of BosR as it serves as the cofactor for BosR binding to DNA (Boylan, Posey, & Gherardini, 2003; Katona, Tokarz, Kuhlow, Benach, & Benach, 2004). Furthermore, the activity of peptide deformylase and glycolytic enzyme fructose-1-6-bisphosphate aldolase depend on zinc (Nguyen, Wu, Boylan, Gherardini, & Pei, 2007). Because glycolysis is the only mechanism used for generation for ATP within B. burgdorferi, zinc is an important metal in B. burgdorferi (B. Troxell & Yang, 2013).

Lastly, there is evidence for a role for copper in the spirochete. B. burgdorferi expresses a metal binding protein called NapA or BicA, a ferritin-like DNA binding-protein that can bind copper. While the mechanism for copper transport is unknown in B. burgdorferi, BicA may be involved in homeostasis of copper (Wang, Lutton, Olesik, Vali, & Li,
The requirement for copper in *B. burgdorferi* as an enzymatic co-factor is currently unknown.

**Calprotectin**

Even though *B. burgdorferi* has no requirement for iron, is the spirochete susceptible to the nutritional immunity response of the host? As mentioned above, high levels of manganese and zinc are important for *B. burgdorferi* survival. Interestingly, the mammalian host has evolved with a means for withholding these metals during infection and inflammation that involves calprotectin and other members of the S100 family of proteins.

The S100 protein family was first identified by B.W. Moore in 1965 (Moore, 1965). It was named S100 because of their solubility in a 100%-saturated solution with ammonium sulfate at neutral pH. S100 proteins are small, acidic proteins of 10-12 kDa and contain two calcium binding EF-hands (Sedaghat & Notopoulos, 2008). This family of proteins is involved in many processes such as proliferation, differentiation, apoptosis, calcium homeostasis, energy metabolism, and inflammation. In humans, there are at least 25 EF hand calcium-binding proteins in the S100 family and these can form homodimers, heterodimers, or tetramers (Chen, Xu, Jin, & Liu, 2014).

One of the best studied members of the S100 family of proteins is calprotectin, a heterodimer consisting of S100A8 and S100A9 (Korndörfer, Brueckner, & Skerra, 2007). This innate immune factor is mostly abundant in neutrophils and plays an important role
in nutritional immunity. It is found at sites of infection in excess of 1mg/ml (Corbin et al., 2008). Calcium binding changes the quaternary structure of calprotectin by assisting in the conversion of the dimer to the tetramer form (Leukert et al., 2006). Although calprotectin has long been known to play a role in inflammation and infection, very recent studies largely conducted by Eric Skaar at Vanderbilt have shown that calprotectin works to withhold zinc and manganese from invading pathogens.

Calprotectin displays two transition metal-ion binding motifs per heterodimer at the interface (Korndörfer et al., 2007). Site 1 (S1) is a His$_4$ motif formed by residues His17 and His27 from S100A8 and His91 and His95 from S100A9. Site 1 can also be a His$_6$ motif with the addition of His103 and His105 from the S100A9 C terminal tail. Site 2 (S2) is a His$_3$Asp motif formed by residues His83 and His87 from S100A8 and residues His20 and Asp30 from S100A9. Both site 1 and 2 bind to zinc with high affinity. However, only the His$_6$ motif of site 1 can bind to manganese with high affinity (Fig. 6) (Damo et al., 2013; Hayden, Brophy, Cunden, & Nolan, 2013). The affinity for these metals increases in presence of calcium due to protein conformational changes (Brophy, Hayden, & Nolan, 2012).

Calprotectin binds two zinc ions per heterodimer with a dissociation constant ($K_d$) of 5.6nM and 1.35nM at sites 1 and 2 respectively, and one manganese ion at site 1 with a $K_d$ of 1.3nM (Brophy et al., 2012; Kehl-Fie et al., 2011). Although site 2 displays the same primary coordination sphere as Mn-containing superoxide dismutases, site 2 binds manganese with low affinity. Furthermore, calprotectin does not bind iron (Damo et al.,
Calprotectin is also shown to bind to copper (Kerkhoff, Vogl, Nacken, Sopalla, & Sorg, 1999).

Overall, calprotectin has potent antimicrobial activity. WT calprotectin showed a dose dependent growth inhibition for a variety of Gram-positive and Gram-negative bacterial pathogens including *S. aureus*, *Staphylococcus epidermis*, *Staphylococcus lugdunensis*, *Enterococcus faecalis*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *E. coli*, and *Shigella flexneri*. Using mutants of calprotectin that affect zinc or manganese binding, Skaar and colleagues were able to demonstrate that most of these organisms were sensitive to manganese withholding properties of calprotectin, not zinc-withholding. For example, the ΔS1 mutant, which only binds zinc, was unable to inhibit growth of the same pathogens listed above (Damo et al., 2013). While these studies seem to indicate that the calprotectin blocks pathogen growth by withholding manganese, there is also literature on calprotectin working to withhold zinc from fungal pathogens such as *C. albicans* (Clohessy & Golden, 1995; Sohnle, Hunter, Hahn, & Chazin, 2000) and *Aspergillus nidulans* (Bianchi, Niemiec, Siler, Urban, & Reichenbach, 2011).

Other S100 proteins may also work to withhold metals. An example of a human specific S100 protein is S100A12, which was discovered in white blood cells and constitutes about 5% of the total cytosolic protein in neutrophils. S100A12 has been shown to bind zinc and copper. S100A12 is capable of inhibiting *S. aureus* growth, but not to the extent of WT calprotectin (Moroz et al., 2009). S100A7, also called psoriasin, is expressed by human epithelial tissues (Brodersen, Nyborg, & Kjeldgaard, 1999). S100A7 has two
canonical binding sites for zinc that are identical to the zinc binding site in calprotectin, although S100A7 would not be predicted to bind manganese (Brodersen et al., 1999; Damo et al., 2013). Even though S100A7 is able to potently inhibit growth of E. coli, it was substantially less effective against S. aureus, S. epidermidis, and P. aeruginosa (Glaser et al., 2005) perhaps reflecting differences in zinc versus manganese dependent pathways in bacterial pathogens.

Prior to my thesis research, one paper reported that B. burgdorferi was susceptible to calprotectin when the spirochetes were grown in BSK II. This susceptibility of calprotectin was reversed upon addition of 3 µM zinc to BSK II and because of this, the authors suggested that zinc depravation is probably the mechanism of action (Lusitani, Malawista, & Montgomery, 2003). However a major caveat to these studies is that such zinc addition will also block manganese withholding by calprotectin because manganese and zinc bind to the same site. Since these studies were published in 2003, there has been much evidence supporting a role for calprotectin in withholding manganese from bacterial pathogens (Damo et al., 2013). Additionally, a number of calprotectin mutants have been generated that block manganese or zinc binding and represent excellent tools to study the metal withholding antimicrobial properties of calprotectin.

In this thesis, I examine in detail the effect of calprotectin on B. burgdorferi growth in culture. We find that B. burgdorferi is sensitive to calprotectin toxicity, but at doses that are much lower than E. coli grown in the same medium. This toxicity involves the zinc-binding site (site 2) of calprotectin as opposed to site 1. We find that S100A12 is also
toxic to *B. burgdorferi* at doses similar to that of calprotectin. Metal analysis of *B. burgdorferi* showed that rather than withholding metals, calprotectin and S100A12 toxicity was associated with increases in manganese, copper, and zinc levels in *B. burgdorferi*. We also find that calprotectin toxicity reflects direct binding of calprotectin to *B. burgdorferi* and this binding requires zinc-binding site 2. We provide a model in which calprotectin inhibits growth of *B. burgdorferi* not by withholding metals but rather by causing cell toxicity by direct binding to the bacteria and inducing uptake of metals.
Figure 1. Reported Cases of Lyme Disease by Year, United States, 1995-2013 (Adapted from [http://www.cdc.gov/lyme/stats/chartstables/casesbyyear.html](http://www.cdc.gov/lyme/stats/chartstables/casesbyyear.html))

According to the Center for Disease Control, the reported cases of Lyme disease has risen from 11,603 confirmed cases in 1995 to 27,203 confirmed cases in 2013.
Figure 2. Reported Cases of Lyme Disease- United States 2001 (Adapted from http://www.cdc.gov/lyme/stats/index.html)

One dot is placed randomly within county of residence for each confirmed case. Cases are reported based on the county of residence.
Figure 3. Reported Cases of Lyme Disease- United States 2013 (Adapted from http://www.cdc.gov/lyme/stats/index.html)

One dot is placed randomly within county of residence for each confirmed case. Cases are reported based on the county of residence.
Figure 4. Cartoon Depicting the Cross Section of a Spirochete

Spirochetes are helically shaped and motile. The outer cell membrane surrounds the protoplasmic cylinder complex, which consists of the cytoplasm, inner cell membrane, and peptidoglycan. They also have flagella that are located in the periplasmic space between the outer cell membrane and the protoplasmic cylinder as shown in the depicted cross section of a spirochete (Barbour & Hayes, 1986).
Table 1. Metal Analysis of BSK II.

<table>
<thead>
<tr>
<th>Element</th>
<th>BSK II (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na</td>
<td>174026.70</td>
</tr>
<tr>
<td>Mg</td>
<td>784.26</td>
</tr>
<tr>
<td>P</td>
<td>3530.49</td>
</tr>
<tr>
<td>K</td>
<td>4908.37</td>
</tr>
<tr>
<td>Ca</td>
<td>1549.41</td>
</tr>
<tr>
<td>Cr</td>
<td>0.26</td>
</tr>
<tr>
<td>Mn</td>
<td>0.18</td>
</tr>
<tr>
<td>Fe</td>
<td>11.87</td>
</tr>
<tr>
<td>Co</td>
<td>0.09</td>
</tr>
<tr>
<td>Ni</td>
<td>0.12</td>
</tr>
<tr>
<td>Cu</td>
<td>5.95</td>
</tr>
<tr>
<td>Zn</td>
<td>3.30</td>
</tr>
</tbody>
</table>

ICP-MS analysis of BSK II. Contributed by Daphne Aguirre.
Figure 5. Tick Life Cycle (Adapted from (Barbour & Zuckert, 1997))

The three stages of a tick are larvae, nymph, and adult tick (J. Piesman et al., 1987). The life cycle of _I. scapularis_ lasts 2 years. During the months of August to September of year one, the larvae feed on mice, birds, and other small animals (Joseph Piesman & Spielman, 1979). During year two in May to July, the nymphs feed either on other small rodents or humans. These nymphs moult to adults that same fall. Adult ticks usually feed on large animals such as deer or humans. The female adults lay eggs and the life cycle continues (Yuval & Spielman, 1990).
Figure 6. Structure of Calprotectin

Crystal structure model of the calprotectin heterodimer displays two transition metal-ion binding motifs per heterodimer at the interface. The heterodimer consists of S100A8 (blue) and S100A9 (purple). Spheres represent the following: Ca = gray; Mn = green; Zn = yellow. Both site 1 and 2 of WT calprotectin can bind to zinc with high affinity. However, only site 1 can bind to manganese with high affinity. Unpublished results from the Culotta lab also indicate that copper binds to site 1 using the same histidines required for manganese binding (contributed by Angelique Besold).
Experimental Procedures

Bacterial strains, growth medium and growth conditions

The *B. burgdorferi* WT strains ML23 and 297 and the *bmtA* mutant were used. The *bmtA* mutant strain, created by allelic exchange in WT strain 297, was provided by M.V. Norgard (Norgard et al., 2009). ML23 was provided by J. Seshu. *E. coli* strain DH5α was used. *B. burgdorferi* was typically grown in BSK II medium (pH 7.6) supplemented with 6% (v/v) rabbit serum (Sigma) also containing 0.05mg/ml rifampicin, 0.1mg/ml phosphomycin, and 5µg/ml amphotericin b (Pollack, Telford, & Spielman, 1993). The concentrations of various metals in BSK II medium are shown in Table 1. *B. burgdorferi* cultures were typically inoculated from frozen stocks at a density of $10^4$ cells/ml and grown at 34°C to a density of $10^7$ to $10^8$ cells/ml. *E. coli* was grown in BSK II medium without antibiotics and at 37°C.

Stocks of *E. coli* cells for testing sensitivity towards calprotectin were obtained by growing overnight at 37°C in 3ml Luria-Bertani broth (Sezonov, Joseleau-Petit, & D'Ari, 2007) in a 15-mL BD Falcon Polypropylene Round-bottom tube on a shaking incubator. The next morning *E. coli* was diluted to an optical density at 600 nm (OD$_{600}$) of 0.02 in 5-ml BD Falcon Polypropylene Round-bottom tubes with 20% calprotectin buffer (20 mM Tris(hydroxymethyl)aminomethane (Tris) pH 7.5, 100 mM Sodium chloride (NaCl), 10 mM β-mercaptoethanol (BME), 3 mM Calcium chloride (CaCl$_2$)), 80% BSK II medium, and various concentrations of WT or mutant calprotectin. *E. coli* was grown at 37°C with shaking at 250 rpm and growth at 5.5 h was examined at OD$_{600}$. Details on calprotectin handling are described below.
For studies of *B. burgdorferi* sensitivity towards calprotectin and S100A12, cultures were inoculated from frozen stocks at a density of $10^4$ cells/ml into 96-well flat-bottom plates that contained 20% calprotectin buffer (20 mM Tris pH 7.5, 100 mM NaCl, 10 mM BME, 3 mM CaCl$_2$), 80% BSK II medium and various concentrations of WT or mutant calprotectin or S100A12. Each well in the plate had total volume of 200 µl. Samples were either in duplicates or triplicates. Control for the experiments included the same amount of BME that would be contributed from calprotectin. The plates were covered with sterile adhesive film covers (Axygen) before putting the plate cover on top. Then, tape was used to seal the edges. The cells were grown at 34°C in the incubator (Fisher Scientific, Isotemp Incubator) without shaking. Growth was examined typically 1 week afterwards. After about 1 week, in the sterile hood, 20 µl from each well was transferred to microfuge tubes. 10 µl of the culture was added to the cut-out notch on Glasstic Slide 10 with Grids (Cova), and observed on the microscope (Nikon Eclipse 80i). Using a counter, the number of spirochetes was counted for three boxes on the glass slide for each sample and averaged. If the number of spirochetes was greater than ~120/box, the sample was diluted and counted as before.

To study the effect of calprotectin and S100A12 on metal accumulation in *B. burgdorferi*, cultures were inoculated from frozen stocks at a density of $10^5$ cells/ml into 50mL Falcon conical centrifuge tubes in 20% calprotectin buffer (20 mM Tris pH 7.5, 100 mM NaCl, 10 mM BME, 3 mM CaCl$_2$), and 80% BSK II medium. The total volume was 20ml in each tube sealed with cap. After growing in the incubator for 2-3 days at 34°C without shaking to a density of $1-4 \times 10^6$ cells/ml, 20ml of cells were
separated into two 10 ml cultures. At this time, 80 µg/ml of calprotectin was added. Culture was then grown again in the incubator for an additional 7-8 days at 34°C to a density of 10^7-10^8 cells/ml. Afterwards, *B. burgdorferi* cells were harvested by centrifugation (Centrifuge 5810R) at 3,000 × g at 4°C for 1 hour in 15ml Falcon conical centrifuge tubes. The pellets were transferred to microcentrifuge tubes and washed twice in cold TE buffer and once in cold milliQ water. As a blank control, the same volume of BSK II medium incubated in parallel but no cells was subjected to the identical centrifugation and washing treatments. For metal analysis, fresh cell pellets were treated with nitric acid each time.

For preparation of *B. burgdorferi* cell lysates, cultures of *B. burgdorferi* were inoculated from frozen stocks at a density of 10^5 cells/ml into 50mL Falcon conical centrifuge tubes in 20% calprotectin buffer (20 mM Tris pH 7.5, 100 mM NaCl,10 mM BME, 3 mM CaCl_2_), and 80% BSK II medium, at total volume of 10ml. After growing for 2-3 days to density of around 10^6 cells/ml, 80 µg/ml of calprotectin was added. Then culture was grown again for about 4 days. They were harvested at ~5 ×10^7 cells/ml by centrifugation (Centrifuge 5810R) in 15ml Falcon conical centrifuge tubes at 3000 × g at 4°C for 1 hour and washed twice in cold washing buffer (20mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) pH 7.60, 100mM NaCl, 10mM EDTA) (Posey & Gherardini, 2000) and once with cold milliQ water.
Calprotectin and S100A12 protein

The purified WT and mutant calprotectin proteins as well as S100A12 were provided by W. Chazin, Vanderbilt. Calprotectin was typically stored in calprotectin buffer (20 mM Tris pH 7.5, 100 mM NaCl, 10 mM BME, 3 mM CaCl2) at -80 °C and in 250 µl to 1 ml aliquots. S100A12 was stored in buffer with 20mM Tris pH 8.0, 100mM NaCl and 10mM BME also at -80 °C in 250 µl to 500 µl aliquots. Prior to use, proteins were thawed on ice and supplemented with CaCl2 to a final concentration 3mM. Proteins were sterilized with SpinX Centrifuge Tube Filter (Costar) before adding to cultures.

Analysis of metals by Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

Cell pellets and blank controls as obtained above were resuspended in 500µl of 67-70% (v/v) nitric acid (J.T. Baker, Ultrace II Ultrapure Reagent) and heated at 80°C for 1 hour in microcentrifuge tubes secured with cap locks. Cell debris was removed by centrifugation (Centrifuge 5430R) for 20,000 × g for 5 min at 4°C. Supernatant was removed and put into a fresh microcentrifuge tube. Samples diluted 1:14 in milliQ water prior and analyzed by ICP-MS in semiquantitative mode (Agilent 7700x, University of Maryland, School of Pharmacy, Mass Spectrometry Center, Nebulizer: MicroMist, RF Power: 1550 W, Ar carrier gas flow rate: 1 L/min, Ar make-up gas flow rate: 0.1 L/min, Reaction gas: Helium, He gas flow rate: 4.5 mL/min, Octopole RF: 160 V, Quadrupole Bias: -15 V, Octopole Bias: -18 V).

As a blank control, the same volume of BSK II medium incubated in parallel but no cells was subjected to the identical centrifugation and washing treatments. The two blank
samples given in µg/L was averaged and subtracted from the *B. burgdorferi* samples for each metal, manganese, copper, and zinc. The final element concentration given in µg/L was converted to nmoles/10⁹ cells based on the cell number that was obtained from counting cells as indicated above.

*Analysis of whether calprotectin binds to B. burgdorferi.*

Cells pellets that were frozen at -80° C or freshly obtained as described above were resuspended in 150 µl of lysis buffer containing 10mM potassium phosphate (KPi) pH 7.8, 5mM ethylenediaminetetraacetic acid (EDTA), 5mM ethylene glycol tetraacetic acid (EGTA), 50mM NaCl, 0.45% Tergitol-type NP-40 (NP40), 10% glycerol. Cells were lysed in a vortex shaker (Vortex-Genie 2, Scientific Industries) using 0.7-mm zirconium oxide beads equivalent to volume of pellet at 3200 RPM (three cycles of 3 min interspersed with 3 min on ice). Lysates were then clarified by centrifugation at 20,000 × g for 10 min at 4°C. The supernatant was transferred to a fresh microcentrifuge tube and stored in -80 °C.

Protein concentration was analyzed using Bradford assay according to manufacturers specifications (Bio-Rad). Of the lysate protein, 2.5 to 8 µg, brought up in lysis buffer, was added to a lane on the gel. The sample with lysate, 5 µl loading dye (NuPage SDS Sample Buffer 4X) was heated to 37 °C for 20 minutes before running on gel. Calprotectin was resuspended in milliQ water before adding 5 µl loading dye (NuPage SDS Sample Buffer 4X). It was heated in same way as samples before running on gel. For Western blot, cells were separated in NuPage 4-12% BisTris Gel and transferred to a
PVDF membrane (Life Technologies). All membranes were blocked with 5% dry milk in 1x Tris-buffered saline and 1% Tween-20 (TBS-T), then incubated with respective antibodies diluted accordingly. Anti-S100A9 antibody (Abcam) was diluted 1:1,000. Alexa Fluor 680 donkey anti-rabbit secondary (Life Technologies) was diluted 1:10,000. Western blots were analyzed using Odyssey Imaging Systems (LI-COR Biosciences).

**Results**

*The toxicity of calprotectin and S100A12 in cultures of B. burgdorferi*

*B. burgdorferi* grown in BSK II medium in culture is sensitive to calprotectin. In Figure 7A, growth is inhibited by > 100 µg/ml or 2.73 µmol/L with the ML23 strain and similar results were seen with the independent 297 strain of *B. burgdorferi* (Fig. 7B). By comparison, *E. coli* grown in the same BSK II medium requires nearly 10 times more calprotectin to show growth inhibition (Fig. 8A), similar to what has been published for *E. coli* grown in tryptic soy broth (Damo et al., 2013).

Growth inhibition of *E. coli* by calprotectin has been published to involve manganese withholding by calprotectin (Damo et al., 2013). Although calprotectin will bind to both manganese and zinc, its sequestration of these two metals can be discerned by specific mutations. The M1 mutant of calprotectin that has H103, H104, and H105 replaced by asparagines cannot bind manganese at site 1 of calprotectin. The M2 mutant of calprotectin has four histidine residues of site 1 replaced by asparagines, 3 histidine residues of site 2 replaced by asparagines, and one aspartic acid residue of site 2 replaced by serine such that it cannot bind either manganese or zinc (Fig. 8B). As seen in Fig. 8A,
the M2 mutant of calprotectin defective for binding both manganese and zinc showed no growth inhibitory effects on *E. coli*. The M1 mutant defective for only binding manganese was also greatly reduced in its ability to inhibit *E. coli* growth but not to the extent of M2 calprotectin (Fig. 8A). Thus, the manganese withholding properties of calprotectin properties are essential for maximal inhibition of *E. coli* growth but zinc withholding may also contribute.

Using these same mutants of calprotectin, we tested whether the growth inhibition of *B. burgdorferi* was dependent on site 1 and/or site 2 metal binding sites of calprotectin. As with *E. coli*, the growth inhibitory properties of calprotectin were totally reversed in the M2 mutant lacking the ability to bind any metals (Fig. 9A). However, unlike results with *E. coli*, the M1 mutant of calprotectin was severely toxic to *B. burgdorferi*, nearly as potent as WT calprotectin (Fig. 9A). The same results were obtained with both the 297 (Fig. 9A) and ML23 (Fig. 9B) strains. Since manganese binding to calprotectin is completely disrupted in the M1 mutant, these results alone would suggest that manganese withholding is not as critical for calprotectin toxicity in the case of *B. burgdorferi*.

Manganese accumulation in *B. burgdorferi* is dependent on the BmtA manganese transporter (Norgard et al., 2009, Aguirre et al., 2013). Thus, we tested whether the pattern of calprotectin toxicity was altered in *bmtA* mutants that accumulated low manganese. As seen in Fig. 10, the *bmtA* mutant is also highly sensitive to toxicity from calprotectin, similar to the parental WT 297 *B. burgdorferi* strain, and this toxicity is totally reversed in the M2 mutant of calprotectin that cannot bind any metals. However,
calprotectin toxicity to the bmtA mutant was substantially reversed by the single M1 mutation that disrupts the manganese binding properties of calprotectin but not the zinc withholding properties (Fig. 10, right). Since the bmtA mutant accumulates extremely low levels of manganese (Norgard et al., 2009, Aguirre et al., 2013), it might be more sensitive to the manganese binding effects of calprotectin.

Another S100 protein is A12 which has been shown to bind to zinc and copper (Moroz et al., 2009). Interestingly our collaborator Andrea Marques at the NIH found that A12 as well as calprotectin is induced at sites of tick bites in patients affected with Lyme disease (personal communications). We find A12 is also toxic to B. burgdorferi at doses similar to that of calprotectin (Fig. 11A). Furthermore, similar to calprotectin, both the WT and bmtA mutant strains of B. burgdorferi are sensitive to toxicity from A12 (Fig. 11B).

We tested whether the effects of A12 and calprotectin on B. burgdorferi were cytotoxic, i.e., cells were dead or cytostatic, i.e., cell growth inhibited but cells were viable. In the experiment of Fig. 11C, the indicated cells were treated with 125 µg/ml of calprotectin (3.42 µmol/L) or A12 (11.7 µmol/L) for 1 week, a concentration that severely inhibits growth (see Fig. 7 and 11B) as indicated in the dark bars of Fig. 11C. Then, cells were diluted ten fold in fresh growth medium and allowed to grow for 10 days. In virtually all cases, full growth was restored to that of control cells that were never treated with calprotectin, indicating that calprotectin and A12 are working to inhibit B. burgdorferi growth, but not kill the bacteria (Fig. 11C). Interestingly, the growth of the bmtA mutant

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treated with A12 was not fully restored to that of untreated cells (Fig. 11C), suggesting some possible cytotoxicity in this case.

The effect of calprotectin and S100A12 on metal accumulation in B. burgdorferi

To address whether calprotectin and A12 were actually working to starve B. burgdorferi of metals, we used Inductively Coupled Plasma Mass Spectrometry (ICP-MS) that can provide information on cellular content of various metals including manganese, zinc, and copper. In the experiments of Fig. 12 and 13, B. burgdorferi were treated with 80 µg/ml calprotectin (2.19 µmol/L) or A12 (7.69 µmol/L) for only 4-5 cell doublings compared to the 10 cell doublings of toxicity studies of Fig. 7, 9-11. With this type of treatment, there was minimal growth inhibition to B. burgdorferi (Fig. 12A and 13A) and sufficient cellular material could be obtained for metal analysis. We strongly anticipated that calprotectin and A12 would decrease accumulation of metals in B. burgdorferi including manganese and zinc but surprisingly, the opposite was observed. Over several experimental trials, treatment with calprotectin resulted in a ≈50% increase in cellular manganese (a representative experiment is shown in Fig. 12B). In addition, there was a large increase (5-10 fold) in cellular zinc (Fig. 12C). Calprotectin has previously been shown to bind copper (Kerkhoff et al., 1999) and we also see a ≈3 fold increase in cellular copper with calprotectin treatment of B. burgdorferi (Fig. 12D). Next, we looked at the cellular content of metals in B. burgdorferi that were treated with the mutants of calprotectin M1, which only binds zinc, and M2, which cannot bind to any metals. The increase in manganese and copper accumulation was prevented by both the M1 and M2 mutants of calprotectin (Fig. 12B,D). On the other hand, the increase in zinc
accumulation was still seen with the M1 mutant, but not with the M2 mutant (Fig. 12C). Thus, the increase in metals seen with calprotectin treated cells completely correlates with the metal binding specificities of calprotectin.

Furthermore, we wanted to look at the content of these metals in the bmtA mutant. As seen with the WT strain of B. burgdorferi, there was an increase in cellular manganese, zinc, and copper in the bmtA mutant treated with calprotectin (Fig. 12B-D). As expected, the bmtA mutant had very low manganese, but treatment with calprotectin brought manganese levels back to that of the WT strain of B. burgdorferi (Fig. 12B). In addition, there was a large increase in zinc about 5-8 fold (Fig. 12C) and ≈3 fold increase in copper with calprotectin treatment of bmtA mutants (Fig. 12D). Therefore, the increase in metals seen with calprotectin treated cells is independent of the BmtA metal transporter.

Next, we wanted to look at the effects of A12 on the content of these metals in B. burgdorferi. Because A12 is known to bind copper and zinc (Moroz et al., 2009), we strongly anticipated that A12 would withhold these metals from B. burgdorferi, but that was not observed. Treatment with A12 also resulted in increased metal accumulation in B. burgdorferi. However, there were some notable differences compared to the effect of calprotectin. The degrees of copper and zinc accumulation in B. burgdorferi were not as high as seen with treatment of calprotectin (Fig. 13C-D). Compared to the 5-10 fold increase of zinc in treatment of calprotectin, there was only about a 2 fold increase in cellular zinc with A12 treatment (Fig. 13D.) In addition, unlike calprotectin, A12 did not restore manganese levels in the bmtA mutant to that of WT strain of B. burgdorferi (Fig. 13B). These differences suggest that calprotectin and A12 are affecting metals to
different extremes although both are equally toxic to \textit{B. burgdorferi} as seen with the growth inhibition assays (Fig. 11A).

\textit{Direct binding of calprotectin to \textit{B. burgdorferi}}

The effects of calprotectin and S100A12 on metals in \textit{B. burgdorferi} was quite surprising. How could these proteins lead to an increase in bacterial metals? As one possibility, \textit{B. burgdorferi} might acquire metals from calprotectin. For example, it has been shown that for \textit{Neisseria meningitidis}, a cell surface protein known as CbpA functions as receptor for calprotectin such that the bacteria can use calprotectin as a source of metal nutrients (Stork et al., 2013). We therefore wished to test whether \textit{B. burgdorferi} also acquires metals from calprotectin by directly binding to this protein. We first demonstrated that a commercial anti-S100A9 antibody was able to detect purified calprotectin (Fig. 14A lanes 1-6). Then, I prepared \textit{B. burgdorferi} cells the same way I had prepared them for ICP-MS in Figs. 12 and 13 and ran a Western blot on \textit{B. burgdorferi} cells that have been treated or not treated with calprotectin. Interestingly, we found that calprotectin could be detected in \textit{B. burgdorferi} cells that have been treated with this protein (Fig. 14A lane 8). By comparing to known amounts of calprotectin (lanes 1-6), we estimated that roughly 110 ng of calprotectin associates with 1.3x10e7 cells (lysate equivalents run on gel), which represents about 0.3 nmoles of calprotectin/10^9 cells.

Next, we wanted to see if the mutant versions M1 and M2 of calprotectin also bind to \textit{B. burgdorferi}. First, we verified that the commercial anti-S100A9 antibody cross-reacted with all three forms of calprotectin shown in top of Fig. 14B. Surprisingly, we find that
M1 calprotectin defective for metal binding in site 1 was associated with *B. burgdorferi* while the M2 mutant lacking both metal sites was not (Fig. 14B). It is important to remember that the M1 mutant cannot bind to manganese or copper but still has the ability to zinc, while the M2 mutant cannot bind to any metals, suggesting that calprotectin’s ability to bind to *B. burgdorferi* maybe related to its ability to bind zinc. More importantly, this metal dependent binding of calprotectin to *B. burgdorferi* completely correlates with calprotectin’s toxic effects to *B. burgdorferi*. As mentioned before, the M1 mutant of calprotectin was severely toxic to *B. burgdorferi*, nearly as potent as the WT calprotectin, while the M2 mutant didn’t have any toxic effects (Fig. 9). Together these findings demonstrate that the metal binding sites of calprotectin allow the protein to directly bind to *B. burgdorferi* resulting in an apparent increase in metal accumulation and cell toxicity.

**Discussion and future directions**

Calprotectin, with its potent antimicrobial activity, has been thought to work by withholding metals (Damo et al., 2013). We find that calprotectin is toxic to *B. burgdorferi* as is S100A12 due to cytostatic and not cytotoxic effects as the growth inhibition is reversible. Originally, we surmised this was due to zinc withholding and starving the bacteria of zinc. The M1 mutant of calprotectin that cannot bind manganese was still quite toxic to WT *B. burgdorferi* and S100A12 has been published to bind zinc not manganese (Moroz et al., 2009). However, much to our surprise, our metal analyses studies showed that rather than starving *B. burgdorferi* of metals, treatment with calprotectin resulted in increases of *B. burgdorferi* manganese, zinc, and copper. Same
trends were observed with S100A12. Copper (≈6 µM), as opposed to manganese (≤ 0.2 µM) and zinc (≈3 µM), is the most abundant metal in BSK II but calprotectin had the largest increase on zinc levels. Manganese (≤ 0.2 µM) is very low in BSK II but calprotectin still enhanced its accumulation in *B. burgdorferi* (Table 1). In the case of calprotectin, these increases correlated with the ability of these metals to bind calprotectin. Manganese is known to bind site 1 but not site 2 of calprotectin and preliminary results in the Culotta lab (Besold, unpublished) show that copper also binds site 1 but not site 2. By comparison, zinc binds both sites 1 and 2. The M1 mutant form of calprotectin that lacks a functional site 1 could no longer increase manganese and copper accumulation in *B. burgdorferi*, but zinc levels still rose with *B. burgdorferi*. Most surprising, we find that the ability of calprotectin to increase metals in *B. burgdorferi* directly correlates with the binding of calprotectin to *B. burgdorferi* cells. Calprotectin remained attached to *B. burgdorferi* even with extensive washing with EDTA metal chelators and NaCl salt, suggesting tight binding.

Although calprotectin is not generally thought to adhere to bacterial surfaces, direct binding of calprotectin to bacteria has been observed in two other cases. For the gram negative *Neisseria meningitidis*, a cell surface protein known as CbpA functions as receptor for calprotectin such that the bacteria can use calprotectin as a source of metal nutrients (Stork et al., 2013). For the gram positive *Finegoldia magna* bacterium, the surface protein L is known to bind calprotectin protein and prevent calprotectin from lysing the bacterial membrane (Akerstrom & Bjorck, 2009). Upon our search of the *B. burgdorferi* databases, we could not identify any homologues to the CbpA protein of *N.*
meningitidis nor the protein L of F. manga; therefore B. burgdorferi must bind calprotectin in a novel mechanism perhaps through some unknown receptor. It is important to note that calprotectin does not universally bind bacteria. For example, calprotectin is toxic to several Staphylococcal and Streptococcal species but does not bind these bacteria (Akerstrom & Bjorck, 2009). Thus calprotectin may inhibit growth of diverse bacterial species through distinct mechanisms. In our future studies, we want to try to understand how B. burgdorferi binds calprotectin and the role of this binding in the bacterial static activity. In order to identify the protein/receptor for calprotectin, we could fractionate B. burgdorferi and see if calprotectin is associated with the cell membrane or is internal to the cell. If it is associated with the outside of the cell as expected, we can biochemically fractionate membrane components and identify those fractions that binds calprotectin, by using pull-down experiments with anti-calprotectin.

We considered the possibility that the increase in metals associated with B. burgdorferi might simply represent metal bound calprotectin that associates with B. burgdorferi. In this case, the metal never really enters B. burgdorferi, rather just associates with B. burgdorferi through extracellular binding of calprotectin. To address this, we calculated the moles of calprotectin binding to B. burgdorferi cells versus the moles of increased metals in calprotectin treated cells. Indeed we find that the numbers correlate in the case of manganese where 0.3 nmoles calprotectin/10^9 cells matches the increase in manganese 0.2 and 0.3 nmoles/10^9 cells, for WT B. burgdorferi and bmtA mutant respectively. However, calprotectin binding of metals outside the cell cannot account for the huge increase in B. burgdorferi zinc and copper observed in calprotectin treated bacteria. Zinc
levels increase by 12 nmoles/10^9 cells while copper increases by 2.5 nmoles/10^9 cells, compared to the 0.3 nmoles bound calprotectin/10^9 cells. Therefore, it seems that the *B. burgdorferi* bacterium is accumulating higher levels of metals in the presence of calprotectin. In future studies, we will test whether the metals are actually internalized in the bacteria. An easy assay is manganese superoxide dismutase (SodA) activity. The *bmtA* mutant has no SodA activity due to very low manganese (Aguirre et al., 2013) and as seen in Fig. 12B, the *bmtA* mutant exhibits near WT levels of manganese when treated with calprotectin. If this manganese indeed entered the cell, it should be enough to activate the SodA, which we can test using SOD activity assays that are standard in the Culotta lab.

Currently, the only metal transporter known to exist in *B. burgdorferi* is BmtA (Norgard et al., 2009). However, the increase in metal uptake with calprotectin and with S100A12 is seen in *bmtA* mutants. Therefore, if the metals are indeed getting inside the cell, they must be using another metal transport system that can operate non-specifically on several metals. As one possibility, this metal transporter may be induced upon interactions of calprotectin with *B. burgdorferi* as part of a stress signal in the bacteria, perhaps in an attempt to counteract the potential metal withholding capacity of calprotectin. As a second possibility, *B. burgdorferi* might be capturing metals directly from calprotectin that bound the cell, similar to what has been published for *Neisseria meningitidis* (Stork et al., 2013).
Finally, why are calprotectin and S100A12 toxic to \textit{B. burgdorferi}? The original hypothesis of metal withholding cannot be correct because if anything metals increase. It is interesting to note that the same M2 mutant that cannot bind metals and does not associate with \textit{B. burgdorferi} is also unable to cause toxicity to \textit{B. burgdorferi}. The M1 mutant that cannot increase manganese and copper but still binds to \textit{B. burgdorferi} does in fact cause toxicity to \textit{B. burgdorferi}. Therefore, toxicity closely correlates with the direct binding of calprotectin to \textit{B. burgdorferi}, not the withholding of metals. In the case of \textit{F. manga}, calprotectin is thought to cause toxicity by disrupting the membrane through direct binding to the microbe and a similar mechanism might take place with \textit{B. burgdorferi} (see model of Fig. 15). In the future, we can test this theory by ultrastructural analysis of \textit{B. burgdorferi} treated with calprotectin, perhaps using electron microscopy.

Overall, my thesis has revealed an unexpected method of calprotectin toxicity to \textit{B. burgdorferi}. Rather than withholding metals as is currently the dogma for calprotectin antimicrobial function (Clohessy & Golden, 1995; Damo et al., 2013; Sohnle et al., 2000), calprotectin treatment results in an increase in metals associated with \textit{B. burgdorferi} and causes toxicity through mechanisms that involve direct binding of the protein to the bacteria.
Figure 7. Inhibition of *B. burgdorferi* growth by WT Calprotectin

*B. burgdorferi* cells were grown in BSKII medium in the presence of indicated concentrations of calprotectin and cell growth determined as described under “Experimental Procedures.” Results represent the averages of duplicate or triplicates cultures; error bars represent range. Strains used: (A) ML23; (B) 297.
Figure 8. Inhibition of *E. coli* growth by WT Calprotectin, M1, and M2 mutants of calprotectin

(A) *E. coli* was grown in BSKII medium in the presence of indicated concentrations of WT calprotectin, M1, or M2 mutants and growth measured as described under “Experimental Procedures.” (B) Structures of WT, M1, and M2 mutants of calprotectin where blue and purple represent S100A8 and S100A9 and green and yellow spheres represent manganese and zinc respectively (contributed by Angelique Besold)
Figure 9. Inhibition of *B. burgdorferi* growth by WT Calprotectin, M1, and M2 mutants of calprotectin

*B. burgdorferi* cells were grown in the presence of indicated concentrations of WT calprotectin, M1, or M2 mutants as in Fig 1. Strains used: (A) 297; (B) ML23.
Figure 10. Inhibition of bmtA mutant growth by WT Calprotectin, M1, and M2 mutants of calprotectin

The bmtA mutant (right) and its parental strain 297 (left) were grown in the presence of indicated concentrations of WT calprotectin, M1, or M2 mutants and growth monitored as described in Figs. 7 and 9.
Figure 11. Inhibition of *B. burgdorferi* growth by S100A12

(A, B). *B. burgdorferi* WT cells (parts A and B) and the bmtA mutant (part B) were grown in the presence of indicated concentrations of WT calprotectin (part A) or S100A12 (parts A and B) and growth determined as in Fig. 1. (C) *B. burgdorferi* strain 297 and bmtA mutant were treated with 125 μg/ml of calprotectin or A12 for 1 week in BSKII where cell growth was ≤10^6 cells/ml (dark bars). Then, cells were diluted ten fold in fresh BSKII medium and incubated for 10 days, after which growth was measured (white bars). Control = cells that were not pre-treated with calprotectin.
Figure 12. Metal Analysis of *B. burgdorferi* Treated with Calprotectin

*B. burgdorferi* strain 297 and the *bmtA* mutant were grown in the presence of 80 μg/ml of WT calprotectin, M1, or M2 mutants for 4-5 cell doublings as described under “Experimental Procedures.” (A) Total cell growth was determined as in Fig. 1. (B-D) Cells were analyzed for the indicated metals by ICP-MS as described under “Experimental Procedures.” Results represent the averages from two independent cultures with the exception of Zn analysis of WT-CP in part C; error bars represent range.
Figure 13. Metal Analysis of *B. burgdorferi* Treated with A12

Growth and metal analysis of WT *B. burgdorferi* versus the bmtA mutant treated with S100A12 was carried out precisely as in Fig. 12. Results represent the averages from two independent cultures with the exception of Mn analysis of bmtA-A12 in part B; error bars represent range.
Figure 14. WT but not the M2 metal binding mutant of calprotectin binds B. burgdorferi cells

Growth of B. burgdorferi strain 297 was carried out as in Fig. 12. (A) Western blot of the indicated amounts of purified WT calprotectin (lanes 1-6) and of whole cell lysates from B. burgdorferi cells untreated or treated with WT calprotectin (lanes 7 and 8). 2.5 μg of lysates or equivalent of ~1.3 × 10^7 cells was added to gel. (B) Western blot on top panel of 100ng of purified WT, M1, and M2 mutants of calprotectin. Western blot on bottom panel of 200ng of WT calprotectin, 300ng of M1 and M2 mutants, and of whole cell lysates from B. burgdorferi cells untreated or treated with WT calprotectin, M1, and M2 mutants. 10 μg of lysates or equivalent of ~2.7 × 10^7 cells was added to gel.
The orange crosses represent WT calprotectin, M1, and M2 mutants of calprotectin as seen in the figure. WT calprotectin can bind to copper (blue circle), manganese (pink circle), or zinc (brown circle) in site 1 and zinc (brown circle) in site 2. M1 mutant can only bind to zinc (brown circles) in both sites. M2 has lost the ability to bind any metals in both sites as seen with the absence of metal binding. In our model, WT calprotectin and the M1 mutant can directly bind to *B. burgdorferi* resulting in the uptake of metals through an unknown pathway. Binding of calprotectin to *B. burgdorferi* also results in calprotectin mediated cytotoxicity. It is currently unknown whether the influx of metals and cytotoxicity are linked or are separate events associated with calprotectin binding.
References


Curriculum Vitae

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