DECIPHERING NOVEL FUNCTIONS OF *BORRELIA BURGDORFERI* SURFACE PROTEINS AND THEIR POTENTIAL ROLES IN BORRELIAL PATHOGENESIS

A Dissertation

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ABSTRACT

Lyme disease is a significant and re-emerging disease that impacts the public health, particularly in endemic regions. The causative agent of Lyme disease, *Borrelia burgdorferi,* is transmitted to humans by *Ixodes* ticks. During tick feeding, *B. burgdorferi* is injected into the skin, and quickly adapts to mammalian environment by differentially expressing a number of genes, many of which encode lipoproteins and contribute to borrelial infectivity. Identification and characterization of these host specific proteins should provide insight into important steps in regard to *B. burgdorferi* colonization as well as borrelial pathogenesis.

In the first part of this study, we identified a previously uncharacterized borrelial lipoprotein designated BBA33. We showed that a *bba33* mutant failed to establish infection in mice and was cleared early in the infectious process, in a manner that could be rescued by genetic complementation with intact *bba33*. In terms of the protein function, we found that BBA33 specifically bound to human type IV and VI collagen *in vitro*. Our findings demonstrated that BBA33 functions as an essential adhesin in experimental infection via its interaction in collagen-enriched tissues.

Previous observation showed that *B. burgdorferi* resists the classical complement-dependent killing; however, the mechanisms of resistance were not described. In the second part of this study, we asked how surface exposed

lipoproteins might promote resistance to the classical complement pathway. Through the use of far-Western immunoblots, we found that the previously identified fibronectin binding protein BBK32 mediates binding to the classical complement C1 complex. Subsequent surface plasmon resonance assays showed that the BBK32 C-terminal domain bound to both C1 and C1r with high affinity. This binding was found to inhibit the activation of C1r. Furthermore, the production and surface localization of BBK32 in a non-virulent, serum-sensitive *B. burgdorferi* isolate increased the bacterial binding to C1 and C1r, and partially promoted bacterial resistance to the classical pathway-dependent killing. This study demonstrated a novel mechanism utilized by *B. burgdorferi* to evade complement system activation and survive in the mammalian host. In addition, we identified a new function of *B. burgdorferi* lipoprotein BBK32 in blocking the classical complement pathway. Given that several pathologies are associated with uncontrolled activation of C1, it is possible that inhibition of this activity by BBK32 derivatives/mimetics may provide therapeutic potential.

Taken together, this study deciphered novel functions of borrelial surface lipoproteins. It also provided insight into the mechanisms of how different surface proteins of *B. burgdorferi* mediate the colonization of host tissues and the evasion of complement-dependent killing. As such, these studies provide additional insight into the role of borrelial surface proteins in the infectivity and pathogenesis of *B. burgdorferi* during experimental infection.

DEDICATION

To my family

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NOMENCLATURE

NHS	Normal Human Serum
TLR4	Toll Like Receptor 4
CDC	Centers for Disease Control and Prevention
LB	Lyme Borreliosis
ORFs	Open Reading Frames
DMC	Dialysis Membrane Chambers
ECM	Extracellular Matrix
FN	Fibronectin
GAGs	Glycosaminoglycans
MAC	Membrane attack complex
SPR	Surface Plasmon Resonance
CP	Classical Pathway
AP	Alternative Pathway
LP	Lectin Pathway

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Borrelia burgdorferi, the causative agent of Lyme disease

Lyme disease was first identified clinically in 1976, when a cluster of children residing in the area of Lyme, Connecticut experienced a mysterious rheumatoid arthritis-like condition (Steere et al., 1977). Subsequently a causal link was established between a tick-based infection and the juvenile arthritis observed. In 1981, in the midgut of hard-body *Ixodes* ticks, Dr. Willy Burgdorfer and colleagues discovered a spirochete, which was designated *Borrelia burgdorferi* and was subsequently identified as the etiologic agent of Lyme disease (Burgdorfer et al., 1982; Johnson et al., 1984). Since then, Lyme disease has transitioned into a significant public health issue in the U.S. and is often plagued by difficulty in treatment particularly in the late stage disease, characterized by fatigue, muscle pain, and arthritis. Lyme disease is the leading vector-borne illness in the United States with approximately 300,000 diagnosed cases per year, according to the Centers for Disease Control and Prevention (CDC) (Hinckley et al., 2014), indicating that Lyme borreliosis is a significant, re-emerging infectious disease.

Phylogenetic analyses categorized Lyme borreliosis (LB) spirochetes into several species, three of which are known to cause Lyme disease in humans The three species are collectively referred to as *Borrelia burgdorferi senso lato (s.l.)*

and include *B. burgdorferi sensu stricto*, *B. garinii*, and *B. afzelii* (Baranton et al., 1992; Margos et al., 2011). *B. burgdorferi senso stricto (s.s.),* referred to as *B. burgdorferi* herein, is found primarily in the United States and western Europe. Normally infection by *B. burgdorferi* results in the formation of a painless rash following the bite of an infected tick, termed erythema migrans, which occurs 3-32 days after infection (Brisson et al., 2012; Radolf et al., 2012). If no treatment is sought, patients can develop neurological illness, arthritis, and carditis (Shapiro, 2014). In Europe and Asia, most tick-borne dermatological manifestations are attributed to *B. afzelii* (Stanek and Strle, 2008). Likewise, neuroborreliosis is associated with *B. garinii* (Stanek and Strle, 2008).

Manifestation and treatment of Lyme disease

Following the bite of an infected tick, *B. burgdorferi* can transmit to the human host resulting in a multi-stage illness characterized by localized, disseminated, and persistent Lyme disease (Steere, 2001). The most common sign of localized Lyme disease is the erythema migrans lesion (Shapiro, 2014; Wormser et al., 2006). Erythema migrans usually appears at the site of the tick bite within 1 to 2 weeks in the form of a small erythematous papule, which subsequently enlarges and persists for 3 to 4 weeks if untreated (Nadelman et al., 1996; Smith et al., 2002; Steere and Sikand, 2003). The typical “bull’s eye rash” appearance of the erythema migrans is attributed by the flux of immune cells including macrophages, dendritic cells and T cells with pro-inflammatory cytokines

(Interleukin-6 and Interferon-γ) (Weis and Bockenstedt, 2010) in response to the dissemination of spirochetes (Salazar et al., 2003). In some patients, erythema migrans is often the sole symptom encountered, but many patients have nonspecific symptoms, including headache, myalgia, arthralgia, fatigue, and, less often, low-grade fever (Nadelman et al., 1996; Smith et al., 2002; Steere and Sikand, 2003).

In the dissemination stage, *B. burgdorferi* is able to migrate hematogenously to other sites in the skin and to extracutaneous sites (Shapiro, 2014). In addition, multiple, often smaller, erythema migrans, are seen at this stage of infection (Shapiro, 2014; Smith et al., 2002). Later, the bacteria spread to multiple tissues and infected individuals can develop musculoskeletal pain, carditis, and neurological symptoms (Duray and Steere, 1988; Steere, 2001; Steere et al., 2004). If untreated, the disease may progress to persistent infection characterized by the aforementioned symptoms. Late, untreated infection is sometimes seen in the form of difficult to treat Lyme arthritis, debilitating neurological symptoms, and chronic fatigue (Halperin, 1998; Logigian et al., 1990; Oschmann et al., 1998; Steere et al., 1987, 1994).

The currently recommended treatment for *B. burgdorferi* includes doxycycline, amoxicillin or cefuroxime axetil, with the clearance being about 90% (Shapiro, 2014; Wormser et al., 2006). It is worth noting that Lyme disease is still highly responsive to antibiotic treatment with early diagnosis in the first two stages of disease (Shapiro, 2014). However, when the infection progresses to the late

phase, treatment can become more difficult. Such patients may suffer from refractory Lyme arthritis, which refers to the inflammation in a large joint (usually the knee) for months or even several years after the spirochetal bacterium is cleared (Feder et al., 2007). Because of these complications and the lack of commercially available vaccines, the best defense is to prevent the initial *B. burgdorferi* transmission or, if infected, to seek antibiotic therapy early in the infectious process.

The enzootic life cycle of *B. burgdorferi*

The enzootic life cycle of *B. burgdorferi* involves hard ticks of the *Ixodes* complex and a diverse species of vertebrates. *B. burgdorferi* is transmitted principally by four species of hard ticks including *Ixodes ricinus* in Europe, *I. persulcatus* in Asia, *Ixodes scapularis* in eastern North America and *I. pacificus* in western North America (Kurtenbach et al., 2006; Radolf et al., 2012). Larvae and nymphal *Ixodes* ticks feed on several small rodent species, such as the white-footed mouse and chipmunk (Levine et al., 1985; LoGiudice et al., 2003), whereas the adult *Ixodes* ticks feed on large mammals, most notably deer. There are three feeding periods during their 2-year life cycle. The larvae ticks are free of *B. burgdorferi* when they hatch, as there is no vertical transmission (Nefedova et al., 2004). The tick species acquire *B. burgdorferi* after feeding on an infected reservoir host, followed by molting into a nymphal tick. Subsequently, the nymph form of the tick seeks a blood meal and, in that process, transmits the pathogen

to the animal or human, usually in the late spring or early summer of the second year. Following the final molt, the adult ticks feed on large animals such as the white-tailed deer during the fall and lay eggs, which are able to persist through the winter and hatch in the following spring or summer into uninfected larvae (Wilson and Spielman, 1985). While the white-tailed deer is required for the life cycle of *Ixodes* ticks, given that they serve as a site for the adult ticks to mate, they are not directly involved in the enzootic life cycle of *B. burgdorferi* as the bacteria do not survive within an infected deer. Humans are an accidental host and there is no documentation of human to human transmission. Most human infections occur in the late spring and early summer, which overlaps with the nymphal feeding period (Radolf et al., 2012).

The basic biology of *B. burgdorferi*

Borrelia spp. are organized similar to Gram-negative bacteria such as *Escherichia coli* with a two-membrane envelope ultrastructure (Samuels, 2010). Unlike conventional Gram-negative bacteria, *B. burgdorferi* lacks lipopolysaccharide in their outer membranes (Takayama et al., 1987) and, as such, do not activate the innate immune response via the toll-like receptor 4 (TLR4) (Hirschfeld et al., 1999; Oosting et al., 2010) . In regard to *B. burgdorferi* envelope structure, a fragile outer membrane overlays the lower protoplasmic cylinder, which consists of peptidoglycan along with a cytoplasmic membrane and the enclosed cytoplasm (Barbour and Hayes, 1986; Johnson et al., 1984). *Borrelia*

burgdorferi is approximately 0.2 to 0.3 μm in diameter by 15 to 20 μm in length and is actively motile with a collection of 7-11 subsurface flagellar organelles located in the periplasmic space (Radolf et al., 2012). The flagella are attached to the poles and wrap around the cell cylinder and, in addition to providing motility, provide the cell with its characteristic flat wave shape (Samuels, 2010).

To date, 28 complete and draft genome sequences of Lyme borreliosis spirochetes have been released (Di et al., 2014; Wattam et al., 2014). Among those, the *B. burgdorferi* strain B31 was the first and probably the most widely used (Casjens et al., 2012; Fraser et al., 1997). The complete strain B31 genome consists of a single linear chromosome of approximately 910 kilobases (kb), and 21 linear and circular plasmids, which represent an additional 600 kb of genetic material. Many of the plasmids are indispensable for *in vitro* growth and infection (Fraser et al., 1997). The chromosome is relatively small compared to other bacteria and *B. burgdorferi* lacks many metabolic synthesis pathways related genes. As such, the Lyme disease spirochete is an obligate parasite. Specifically, *B. burgdorferi* does not synthesize any nucleotides, amino acids, and fatty acids (Fraser et al., 1997). Therefore, the organism is only able to survive by utilizing metabolites provided by its tick vector or mammalian hosts via direct scavenging.

On the other hand, *B. burgdorferi* produces a large number of surface exposed lipoproteins that respond to the environmental changes during its life cycle and interact with different host proteins. The *B. burgdorferi* strain B31 genome is well known for its large number of open reading frames (ORFs)

encoding putative lipoproteins, including 5% of the chromosomal ORF's, and in total 14.5% to 17% of the ORFs located on the plasmids (Fraser et al., 1997). In contrast, *Helicobacter pylori* encodes a total of 20 putative lipoproteins in its 1.67 million base pair genome (1.3% of its coding sequences) (Fraser et al., 1997; Tomb et al., 1997). Borrelial lipoproteins are distributed throughout the inner and outer leaflets of the outer-membrane and the outer leaflet of the inner-membrane (Kenedy et al., 2012). The abundance of lipoproteins as well as their surface exposure suggests that these proteins play an important role in the life cycle of *B. burgdorferi*. The expression of many lipoprotein-encoding genes is augmented in feeding ticks and in mammalian hosts, in response to elevated temperature, nutrient availability, as well as reduced pH (Liang et al., 2002; Samuels, 2011), suggesting that many play an important part in the transmission from the tick vector to the mammalian host. A large number of lipoproteins encoded by these up-regulated genes are related to mammalian infection, such as *ospC*, *dbpBA*, and *bbk32*, and many are required for optimal mammalian infection (Radolf et al., 2012; Weening et al., 2008; Seshu et al., 2006; Grimm et al., 2004; Pal et al., 2004a; Hyde et al., 2009; Samuels, 2011). In addition to the identified pathogenesis-associated genes, several additional uncharacterized genes are regulated in a similar manner (Caimano et al., 2007; Kumar et al., 2010), suggesting that they may serve a function within the context of mammalian infection. Thus, deciphering these "genes of unknown function" in *B. burgdorferi* infection may provide new insight into *B. burgdorferi* pathogenesis.

Transmission of *B. burgdorferi* from ticks to mammalian hosts

Tick attachment starts by inserting the saw-like chelicerae into the epidermis of the host to create an entrance wound (Richter et al., 2013). During attachment, *Ixodes* ticks inject saliva, which contains a mixture of bioactive chemicals, including histamine binders, cytokine and complement inhibitors, and anticoagulants (Nuttall and Labuda, 2008). These chemicals facilitate the tick's ability to ingest blood by providing an anti-coagulant and concomitantly benefit the survival and dissemination of *B. burgdorferi* in mammalian host by muting the host innate immune response (Nuttall and Labuda, 2008).

The *Ixodes* tick engorges on the vertebrate host for 4 to 8 days until it feeds to repletion (Cupp, 1991). During the blood meal, *B. burgdorferi* migrate from the midgut to the salivary gland and then transmit to the epidermal/dermal layer of mammalian host. When an *Ixodes* tick starts feeding on a mammal, the tick midgut environment goes through a dramatic alteration. The ingestion of blood alters the temperature, pH, and availability of nutrients in the midgut and exposes the bacteria to host immune factors such as antibodies and complement proteins, as well as reactive oxygen and nitrogen intermediates as a byproduct of increased tick respiration. Despite these potential inhibitory factors, *B. burgdorferi* rapidly replicates in the midgut during tick feeding (Piesman et al., 1990). In infected nymphs, the number of *B. burgdorferi* increases from fewer than 1000 per midgut prior to feeding to approximately 6000 following 72 hours of attachment (Piesman et al., 2001). Meanwhile, the spirochetes in the tick salivary glands increase >17-

fold, yielding approximately 20 spirochetes per salivary gland pair at 72 h post-attachment when infectivity is at its peak, from approximately 1 per salivary gland pair before feeding. (Ohnishi et al., 2001; Piesman et al., 2001). These observations suggest that feeding ticks provide a stable site for *B. burgdorferi* replication, which is followed by activation and transmission. This process is mediated in part by borrelial surface lipoproteins since the profile of these proteins is dramatically altered during this process (Caimano et al., 2007; Kumar et al., 2010; Ohnishi et al., 2001).

Before the blood meal, *B. burgdorferi* lies in a quiescent state within the tick midgut. At this point, the borrelial cells produce OspA on their surface that serves as an adhesin to the tick protein designated TROSPA (Pal et al., 2004b). Once the blood-meal begins, the *B. burgdorferi* begins to down-regulate *ospA* and express OspC (Ohnishi et al., 2001). Within the overall population the per cell response is variable in this regulation, resulting in some variation of surface exposed OspA and OspC, i.e., some cells are $OspA^-$ and $OspC^-$, some are $OspA^+$ and $OspC^-$, while others are $OspA^-$ and $OspC^+$. Those that fall into latter category are more readily transmitted to mammals (Ohnishi et al., 2001), based on mutant *ospC* strain analysis (Grimm et al., 2004; Pal et al., 2004a).

Gene regulation in *B. burgdorferi*

To quickly adapt to the altered environment and survive in the mammalian host, *B. burgdorferi* regulates its gene expression during transmission (Liang et

al., 2002; Samuels, 2011), including genes that encode infection-associated lipoproteins (Grimm et al., 2004; Hyde et al., 2011a; Maruskova et al., 2008; Pal et al., 2004a; Seshu et al., 2006; Weening et al., 2008; Zhang et al., 1997; Zhi et al., 2015). The Rrp2-RpoN-RpoS regulatory pathway, along with BosR (Hyde et al., 2009; Ouyang et al., 2009), plays a critical role in this process as this regulatory cascade is required for the expression of virulence related genes, including *ospC*, *dbpBA*, and *bbk32* (Radolf et al., 2012; Weening et al., 2008; Seshu et al., 2006; Grimm et al., 2004; Pal et al., 2004a; Hyde et al., 2009, 2010; Samuels, 2011; Ouyang et al., 2009; Hubner et al., 2001). To better understand the transition in gene expression between *in vitro* culture and *in vivo* conditions, Akins et al. developed a new animal model for studying spirochetes in a mammalian host-adapted state (Akins et al., 1998). To accomplish this, *B. burgdorferi* was placed within dialysis membrane chambers (DMC) and implanted into the peritoneal cavity of rats (Akins et al., 1998). After several days the DMCs were removed and the cells evaluated for protein content relative to organisms grown under various *in vitro* conditions (Caimano et al., 2007). These studies showed that, in addition to the aforementioned genes (e.g., *ospC*, *dbpBA*, and *bbk32*), a large number of hypothetical genes were regulated in a similar RpoS-dependent manner (Caimano et al., 2007; Kumar et al., 2010).

RpoS is an alternative RNA polymerase σ-factor, which is transcribed by another alternative σ-factor, RpoN (also known as σ^{54}). Regulation of the *B. burgdorferi rpoS* locus is a remarkably elaborate process. The borrelial *rpoS* gene

is expressed as both temperature and CO_2 levels increases and pH decreases, a condition believed to mimic the tick feeding to mammalian transmission step (Carroll et al., 1999; Hyde et al., 2007; Samuels, 2011; Stevenson et al., 1995). These environmental changes result in an increase in *rpoS* transcripts and subsequent RpoS protein production (Burtnick et al., 2007; Caimano et al., 2004; Hubner et al., 2001; Hyde et al., 2007, 2009; Lybecker and Samuels, 2007; Samuels, 2011; Yang et al., 2000). RpoN-dependent transcription of *rpoS* is mediated by the formation of an *rpoS* promoter complex, including a phosphorylated version of the response regulatory protein 2 (Rrp2) and a Fur/PerR orthologue known as the *Borrelia* oxidative stress regulator or BosR (Boylan et al., 2003; Hyde et al., 2009; Ouyang et al., 2009; Samuels, 2011). BosR acts as a transcriptional activator by binding to the *rpoS* promoter at three regions including one immediately upstream of the RpoN-dependent promoter (Ouyang et al., 2014). Besides Rrp2 and BosR, BadR (BB0693) is also identified as a negative regulator that controls growth phase-dependent induction of *rpoS* and *bosR* in *B. burgdorferi* (Miller et al., 2013; Ouyang and Zhou, 2015). Once *badR* is inactivated, *rpoS* and *bosR* expression is induced only during the early stages of bacterial growth, but not during the stationary growth phase (Ouyang and Zhou, 2015).

Taken together, substantial evidence demonstrates that gene regulation in *B. burgdorferi* is complex at the interface of the tick vector and the mammalian host during transmission (Samuels, 2011). The complicated gene regulation

network in *B. burgdorferi* indicates that borrelial gene expression is strictly controlled and dynamically altered during the enzootic cycle. In addition, these data suggest that differentially regulated genes may play spatial and temporal specific roles during the infection. Therefore, by analogy, determining the functions of additional RpoS-regulated genes of unkown function may provide futher insight into the pathogenic potential of *B. burgdorferi*.

The role of borrelial lipoproteins in colonization and dissemination within the mammalian host

After infection, *B. burgdorferi* colonizes connective tissue in the skin and penetrates deep into the dense irregular connective tissue of the dermis, as well as the dense regular structures within tendons (Barthold et al., 1991, 2006; Cadavid et al., 2003; Pachner et al., 1995). In this process, *B. burgdorferi* interacts with different structural targets within the extracellular matrix (ECM), mediated by borrelial surface proteins (Cabello et al., 2007). For instance, BmpA and ErpX engage laminin (Brissette et al., 2009a; Verma et al., 2009); P66 and BBB07 adhere to integrin proteins (Behera et al., 2008; Ristow et al., 2012); and CspA and CspZ bind to complement proteins, fibronectin (Fn), laminin, and collagens (Hallström et al., 2010, 2013; Kenedy et al., 2009; Rogers and Marconi, 2007). Several additional ECM binding proteins are associated with borrelial pathogenesis. For example, the lipoproteins DbpA and DbpB bind decorin and glycosaminoglycans (GAG's) (Blevins et al., 2008; Fischer et al., 2003; Guo et al.,

1995; Weening et al., 2008). The importance of DbpB/A in borrelial pathogenesis was established when a deletion of *dbpBA* resulted in reduced infectivity in mice (Weening et al., 2008). In addition, a fibronectin and GAG binding protein, designated BBK32, is required for the initial tethering of the bacterium to the endothelium via Fn binding (Fischer et al., 2006; Kim et al., 2004; Norman et al., 2008; Probert and Johnson, 1998; Seshu et al., 2006). This transient interaction results in more stable interactions between BBK32 and GAGs, leading to the dragging of the bacterium along the endothelium, followed by stationary adhesion (Moriarty et al., 2012). More recently, it was shown that BBK32 promotes joint colonization via GAG binding, indicating an additional critical role for BBK32 in borrelial pathogenesis (Lin et al., 2014).

B. burgdorferi is also known to align with host collagen (Barthold et al., 1991; Zambrano et al., 2004). Collagens are ubiquitous within a vertebrate host. For instance, collagen type I and IV are mainly found in skin, while collagen type II is enriched in cartilage, both of which are a preferred colonization niche for *B. burgdorferi* (Lindblad and Kormos, 1991; Shoulders and Raines, 2009). Collagen type VI is another important extracellular matrix protein. It is enriched in skeletal muscle, papillary dermis immediately below the dermal-epidermal junction, and around blood vessels (Watson et al., 2001). Intriguingly, after *B. burgdorferi* infection, the bacteria are extensively found enriched in all the aforementioned tissues of different mammalian hosts (Barthold et al., 1991, 2006; Cadavid et al., 2003; Pachner et al., 1995). Binding to collagen enriched tissues would

presumably not only benefit borrelial colonization, but may also facilitate other aspects of borrelial pathogenesis. For example, the colonization of *B. burgdorferi* around the blood vessels may promote dissemination into the circulatory system (Barthold et al., 1991, 2006; Cadavid et al., 2003; Pachner et al., 1995). Collectively, these findings suggest that the ability to recognize specific collagen types by individual *B. burgdorferi* proteins may contribute to borrelial tissue tropism, promote dissemination, and enhance long-term survival. However, the mechanism of interaction between *B. burgdorferi* and mammalian collagens is not defined. Therefore, the identification of borrelial proteins that bind to particular collagen types may provide important insight into borrelial infectivity strategies and pathogenic correlates.

In addition to the previously described pathogenesis-associated genes such as *ospC*, *dbpBA*, and *bbk32*, several other *B. burgdorferi* genes are known to be regulated in a similar manner (Caimano et al., 2007; Kumar et al., 2010; Yang et al., 2003). However, the function of the proteins encoded by these genes is not known. One such RpoS-dependent gene is *bba33*, which is found on the 54 kilobase (kb) plasmid of *B. burgdorferi* strain B31 (Fraser et al., 1997). Caimano *et al.* showed that *bba33* was up-regulated 29-fold in implanted dialysis membrane chambers (DMC) and 8 fold *in vitro* in an RpoS-dependent manner (Caimano et al., 2007), suggesting that BBA33 is involved in the mammalian side of the infectious lifecycle. Consistent with this contention, *bba33* is also induced during a blood meal in ticks and expressed in infected mouse skin (Kumar et al.,

2010). Furthermore, microarray and RT-PCR experiments from infected non-human primates revealed that *bba33* was significantly up-regulated in heart tissue, but poorly expressed in the central nervous system (Narasimhan et al., 2003), suggesting that *bba33* expression is regulated spatially and thus may contribute to the dissemination and secondary colonization of *B. burgdorferi* at distinct sites within mammalian hosts. Taken together, these data suggested that the *B. burgdorferi* BBA33 protein was involved in some, as yet unknown, mammalian host specific step in borrelial colonization and/or pathogenesis.

The mammalian complement system

The complement cascade represents an important part of the mammalian innate immune response and serves as a first line of defense against pathogens. It also serves as a functional bridge between the innate and adaptive immune responses (Ricklin et al., 2010). One important role of the complement system is to recognize and clear invading pathogens. In this regard, some complement factors are able to opsonize the pathogen, others enhance phagocytosis, while others promote leukocyte chemotaxis. The complement system consists of the classical, the alternative, and the lectin pathways, all of which lead to the formation of the membrane attack complex (MAC) in the cellular membrane and result in the lysis of the organism. The complement system is summarized in **Fig. 1** (Dunkelberger and Song, 2010).

Different complement pathways are activated by recognizing distinct ligands on pathogens. Initiation of the classical pathway is dependent on the C1 complex activation by binding to pathogen-bound immunoglobulins and serum components such as serum amyloid P (SAP) and C-reactive protein (CRP) (Brown et al., 2002; Clas and Loos, 1982; Yuste et al., 2007). The C1 complex consists of one molecule of C1q, two of C1r and two of C1s ($C1q/C1r_2/C1s_2$). The C1q molecule is a 460 kDa glycoprotein composed of six globular “heads”, which are held together by their six collagen-like “stalks”. Each head includes three distinct chains of polypeptide A, B and C that form a collagen-like strand with a triple-helical fibril structure. C1r and C1s are both serine proteases and share similar overall structural organizations. Both proteins contain dual CUB modules, an epidermal growth factor (EGF)-like module, two contiguous complement control protein (CCP) domains, and a chymotrypsin-like serine protease (SP) motif (Morikis and Lambris, 2005). Dimers of the C1r and C1s subcomponents amplify the enzymatic activities associated with the C1 complex and form a tetrameric proenzyme structure around the collagen-like strands of C1q. The binding of C1 with its activators leads to a conformational change of C1q, which triggers the auto-activation of C1r. Subsequently, C1r activates C1s and the latter cleaves C2 and C4 to form the activation products C2a, C2b, C4a, and C4b. C2a and C4b bind to the surface and form the C3 convertase of the classical pathway, deemed C4b2a. C4b2a subsequently cleaves C3 into the anaphylatoxin C3a and the opsonin C3b (Ricklin et al., 2010).

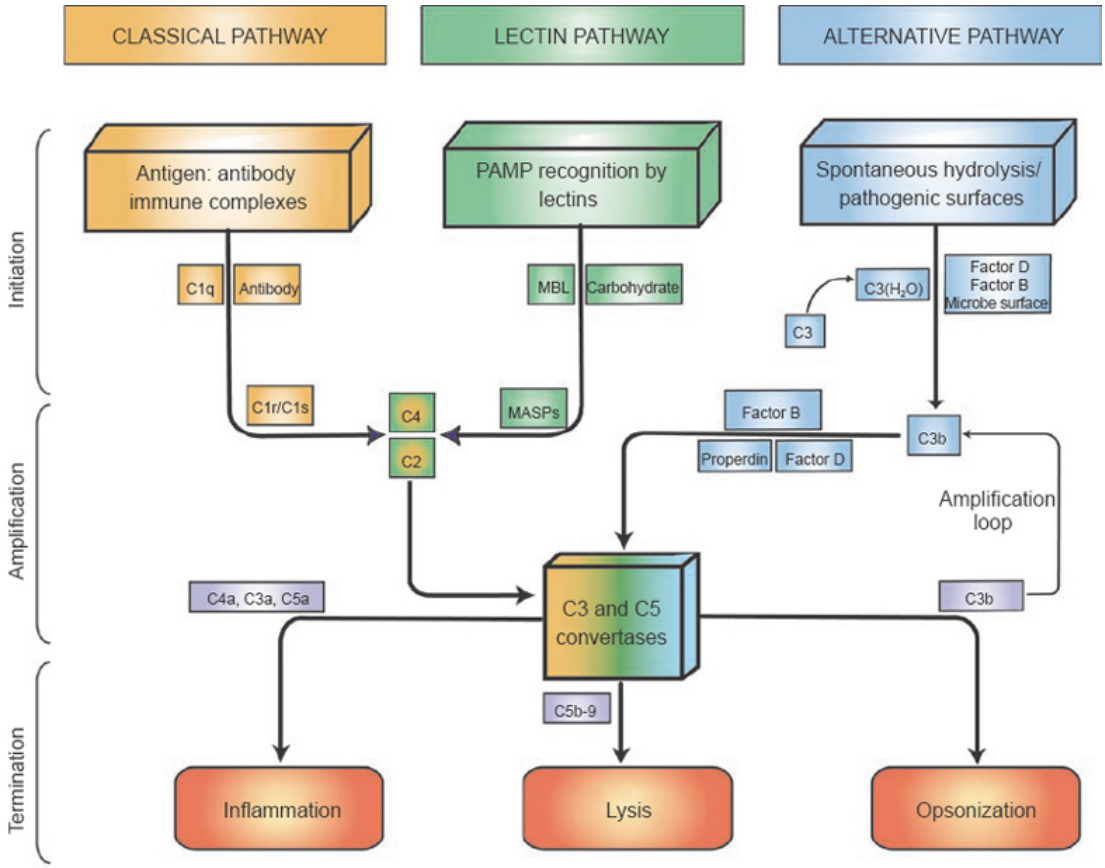

Figure 1. A chematic representation of the complement pathway. The complement system can be activated through three pathways: (1) the classical, (2) the lectin, and (3) the alternative. This figure is reprinted with permission from Dunkelberger and Song, 2010.

The activation of the lectin pathway relies upon binding of the mannose-binding lectin (MBL) or ficolins (FCNs) to highly glycosylated pathogen-associated molecular patterns (PAMPS) on the surface of the pathogen. MASPs 1, 2 and 3 form a complex with MBL or FCNs and become autoactivated after ligand binding. Similar to C1s, activated MASP2 cleaves C2 and C4, to form C3 convertase C4bC2a (Ricklin et al., 2010).

The activation of the alternative pathway (AP) is mechanistically distinct in comparison to the lectin and classical pathways activation. On all cell surfaces, a low level initiation of the alternative pathway occurs spontaneously and continuously through hydrolysis of C3 to C3b(H_2O). Factor B binds to C3b(H_2O) and is subsequently cleaved by factor D to form an initial solvent-based C3 convertase C3bBb, which then cleaves C3 to generate C3b on the cell surface. The C3 convertase is further stabilized by properdin, which helps to amplify the activation of the alternative pathway . As a consequence, the activation of the complement system is exponentially amplified due to the formation of a feedback amplification loop. All three pathways converge at the level of C3, which is cleaved by C3 convertase to generate C3b. C3b covalently binds to the pathogen surface near the activation site to form the C5 convertase and eventually leads to the formation of membrane attack complex (MAC) and lysis of the cell via recruitment of the C6, C7, and C8 proteins and the assembly of multiple C9 proteins.

Regulation of complement activation

Complement activities need to be controlled to the surface of invading organisms, and strictly regulated to prevent collateral damage to host tissues, because of the potential destructive side-effect of complement activation. Hence, multiple steps involved in complement activation are regulated by various inhibitors such that the whole complement system maintains an intricate, homeostatic balance between the minimization of tissue damage and opsonization and destruction of pathogens (Mollnes et al., 2002).

Complement regulators are categorized into soluble and membrane-bound regulators (Cho, 2015). Soluble regulators include factor H, factor H-like proteins, factor H-related protein 1, properdin, C1 inhibitor (C1-INH), C4 binding protein (C4BP), and vitronectin, and are distributed in the plasma and other body fluids (Cho, 2015). Some of these soluble regulators can also attach to cell surfaces and cell membranes to control complement activation in tissues (Rodríguez de Córdoba et al., 2004).

One important soluble regulator of the complement cascade are proteins that belong to the factor H family. This family includes factor H, complement factor H like protein 1 (CFHL1), and five factor-H related proteins (Rodríguez de Córdoba et al., 2004). Factor H is a 150-kDa single-chain plasma glycoprotein that specifically regulates the alternative pathway. Factor H regulates the activation of complement by three different mechanisms: 1) factor H inhibits the interaction of C3b and factor B, thereby blocking the formation of C3bBb; 2) it

contributes to the dissociation of the C3 convertase; and 3) it is a cofactor for factor I, which cleaves C3b (Rodríguez de Córdoba et al., 2004). Properdin is another soluble regulator which serves as an activator of the alternative pathway by binding to C3b to stabilize the complement convertase and prevent its cleavage by factor I (Lesher et al., 2013; Zipfel and Skerka, 2009).

C1-INH and C4BP are two other fluid-phase regulators specifically associated with the classical and lectin pathways. C1-INH is a member of the serine protease inhibitor family. It irreversibly inhibits the classical pathway by disassembling the C1 complex and inactivates MASP1 and MASP2 to block the lectin pathway. C1-INH has been proposed to be useful in animal models to control unnecessary and harmful inflammation, such as Gram-negative sepsis, myocardial reperfusion injury, and hyperacute transplantation rejection (Cho, 2015).

C4BP is a 570kDa soluble glycoprotein that protects many host tissues from damage by complement. C4BP and factor H have similar action (Blom et al., 2004; Zipfel and Skerka, 2009). Like factor H, it has both cofactor and decay-accelerating activity (Gigli et al., 1979). Instead of C3, however, the primary target of C4BP is C4bC2a, although C4BP has also been reported as a cofactor for factor I for C3b cleavage (Blom et al., 2004). In addition, other soluble complement regulators, including vitronectin, clusterin, and CFHR1, target the terminal steps of the pathway, and thus inhibit the formation of MAC on the cell surface. (Heinen et al., 2009; Preissner and Seiffert, 1998; Schwarz et al., 2008).

Host cells also express membrane-associated factors that protect them from the detrimental activation of complement. For example, CD46 is a ubiquitously expressed protein that serves as a co-effector of factor I to cleave C3b and C4b (Andrews et al., 1985). CD59 is a widely expressed small (20 kDa) glycosylphosphatidylinositol (GPI) anchored complement regulator, which blocks C9 from incorporating into the C5b-8 complex as well as C9 polymerization in a pre-formed C5b-9 complex (Meri et al., 1991; Morgan et al., 2005). All of these complement regulators are involved in regulating the activation of the complement system, keeping the balance between destroying foreign cells and minimizing host tissue damage. However, they can also be utilized by pathogens to manipulate and evade host immune systems for optimal infection.

Bacterial evasion strategies against complement activation

Microbes are often recognized and cleared by the immune system of the human host in part via the action of complement. However, some pathogens can survive in an immune-competent human host based on their ability to efficiently inactivate or evade the innate immune response. To successfully infect, pathogenic microbes often utilize numerous, sometimes redundant, and highly complicated strategies to control, modulate, and block complement activation, as well as other innate and adaptive immune responses of the hosts they infect (Blom et al., 2009; Lambris et al., 2008; Rooijakkers and van Strijp, 2007; Zipfel et al., 2007).

Pathogenic microbes interfere with complement activation at different levels. For instance, some bacteria bind plasminogen, the human pre-protease, on their surface and utilize either bacteria-encoded or host activators like urokinase-type plasminogen activator (uPA) to produce the proteolytically active plasmin, which then can cleave and degrade C3 to block its functions (Barthel et al., 2012; Lähteenmäki et al., 2001; Zipfel et al., 2013). *Streptococcus pyogenes* expresses at least five plasminogen binding proteins to promote the evasion of complement activation (Berge and Sjobring, 1993; Pancholi and Fischetti, 1998; Ringdahl and Sjöbring, 2000; Sanderson-Smith et al., 2007). Of note, several borrelial proteins bind to plasminogen as well and may contribute to complement evasion. For example, BBA70-bound plasmin was able to degrade the central complement proteins C3b and C5 (Koenigs et al., 2013). ErpP, ErpA, and ErpC also bind to human plasminogen (Brissette et al., 2009b), but the contribution of this interaction to complement evasion is still not clear.

Infectious bacteria may also directly block the activation of complement early in the cascade. The collagen binding protein, Cna, of *Staphylococcus aureus* binds to C1q and disassembles the C1 complex (Kang et al., 2013). In addition, some Gram-negative bacteria are able to recruit serum amyloid P (SAP) on the cell surface to interfere with LPS mediated activation of the classical pathway (Haas et al., 2000).

The most common soluble complement regulators recruited by different pathogens include factor H, other members of the factor H-like protein family, and

C4BP (Blom et al., 2009; Lambris et al., 2008; Simon et al., 2013; Zipfel et al., 2007). These complement regulators attach to the microbial surface to control the activity of C3 convertase, as well as the amplification loop, eventually leading to complement inactivation (Zipfel et al., 2007, 2013). Alternatively, other pathogens use other strategies to neutralize complement-dependent inactivation/killing. For example, instead of binding to factor H and C4BP, *Bordetella pertussis* binds to C1-INH and neutralizes both the classical and the lectin pathways (Marr et al., 2007). In addition, a large set of pathogenic microbes, including Gram-positive and Gram-negative bacteria, as well as the pathogenic yeast *C. albicans,* bind to vitronectin (Singh et al., 2010). Vitronectin is used by those pathogens to inhibit the terminal steps of complement, which blocks the formation of the membrane attack complex (MAC) on the cell surface, thereby preventing lysis and cell death (Singh et al., 2010).

Complement evasion strategies of *B. burgdorferi*

B. burgdorferi is an extracellular pathogen that can disseminate through both the circulation and lymphoid systems. During the process of dissemination, it is presumed that *B. burgdorferi* must combat the lethal effect of complement to survive. It would then follow that *B. burgdorferi* requires strategies to evade or block complement activation in order to establish and maintain infection.

All of the three complement pathways are involved in complement activation by *B. burgdorferi* (de Taeye et al., 2013). However, different genospecies of Lyme

disease spirochetes differ in their ability to resist complement dependent killing in normal human serum *in vitro* and are categorized as either serum-sensitive or serum-resistant isolates (Breitner-Ruddock et al., 1997; Dam et al., 1997). For instance, *B. burgdorferi*, *B. afzelii*, and *B. garinii* serotype 4 (also known as *B. bavariensis*) are moderately resistant to complement dependent killing *in vitro*, whereas *B. garinii* serotype 5 and 6 are susceptible. Although it is still not clear why different Lyme disease spirochetes confer distinct levels of resistance to human serum, it is postulated that borrelial adaptation to a variety of vertebrate hosts provide a different array of surface proteins that contribute to the variable serum sensitivities observed (de Taeye et al., 2013). In this regard, ticks feed on different vertebrate animals including birds, rodents, and large mammals and transmit *B. burgdorferi* to those animals. In Europe, bird species are the main host for *B. garinii*, whereas *B. afzelii* and *B. bavariensis* infect rodents species (Kurtenbach et al., 2002). *B. burgdorferi* is carried by both birds, rodents, and some larger animals including dogs (Kurtenbach et al., 2002). Correspondingly, *B. garinii* strains, although sensitive to most mammalian serum, are resistant to the killing by bird serum. *B. afzelii* strains are resistant to killing in rodent, human, dog, and cat serum, but are sensitive to the serum from birds. In contrast, *B. burgdorferi* strains are intermediately resistant to serum from humans, rodents, birds, dogs, cats, and horses (Bhide et al., 2005; Kurtenbach et al., 1998, 2002). The pattern of *in vitro* serum sensitivity of different *Borrelia* species mentioned above reflects the reservoir status of many vertebrate species. (Bhide et al., 2005;

Kurtenbach et al., 1998, 2002). For example, large animals, such as deer and cows, are dead-end hosts for all *Borrelia* species, probably due to the spirochetes' inability to prevent complement-dependent killing (Bhide et al., 2005). These observations imply that different Lyme disease spirochetes have evolved to adapt to their main host reservoirs in a manner that avoids complement dependent killing. When these spirochetes are transmitted to humans, an accidental host, the resistance to serum-killing is seen as variable. Further analysis is required to determine how *Borrelia* species specific factors contribute to these variable serum sensitive phenotypes.

A set of borrelial genes that are up-regulated during mammalian infection are involved in resistance to complement dependent killing (de Taeye et al., 2013). Studies of the serum dependent killing first led to the identification of *B. burgdorferi* proteins that bind factor H (FH), factor H-like protein-1 (FHL-1), and FH related protein 1 (FHR-1) (Kraiczy et al., 2001, 2002). These proteins are also referred to as complement regulator-acquiring surface proteins (CRASPs), which include CspA (CRASP1), CspZ (CRASP2), and OspE-related proteins (CRASP-3, -4, and -5) (Hartmann et al., 2006; Hellwage et al., 2001; Kraiczy et al., 2004). To better understand how CspA contributes to serum resistance in *B. burgdorferi*, Brooks et al. and Kenedy et al. generated *B. burgdorferi cspA* mutants and showed that they were susceptible to human serum-dependent killing *in vitro* (Brooks et al., 2005; Kenedy et al., 2009). Furthermore, production of CspA in *B. garinii* 50, a serum sensitive strain, restored the serum resistance to a level indistinguishable

from wild-type *B. burgdorferi* (Brooks et al., 2005). These studies showed that *cspA* mutants do not efficiently bind human FH on their surface, which led to enhanced binding of C3, C6 and C5b-9 onto the surface of the *cspA* mutant relative to that of the wild-type strain (Kenedy et al., 2009). Clearly, CspA contributes to serum-resistance of *B. burgdorferi*, but there are few studies directly showing that CspA specifically inhibits the activation of the alternative pathway. In addition, the importance of the alternative pathway in controlling *B. burgdorferi* infection is controversial as well. In the presence of C1-inhibitor (C1-INH), which inhibits both the classical and lectin pathways but not the alternative pathway, human-serum dependent killing of serum-sensitive *B. garinii* strain was completely abrogated (Schuijt et al., 2011). This results suggest that the alternative pathway may only be important for amplification of complement activation (de Taeye et al., 2013).

In addition to recruiting factor H on the borrelial surface, some *Borrelia* species also develop other strategies to evade complement-dependent killing. For instance, *B. burgdorferi* and the relapsing fever spirochetes *B. recurrentis* and *B. duttonii* recruit C4BP and C1-INH through the interaction of specific surface lipoproteins (Grosskinsky et al., 2010; Meri et al., 2006; Pietikäinen et al., 2010). Also, *B. burgdorferi* expresses a CD59-like protein that inhibits the formation of the lytic MAC (Pausa et al., 2003). In addition to binding to factor H, CspA of *B. burgdorferi* also binds to complement protein C7, which inhibits the terminal complement pathway (Hallström et al., 2013).

Taken together, *Borrelia* evades complement system in various ways to generate serum-resistance to promote infection in mammalian hosts. However, details needed to understand *B. burgdorferi*-complement interactions are still vague due to the lack of *in vivo* data that supports the utility of specific complement proteins in *B. burgdorferi* infectivity and pathogenesis. Of note, complement killing of serum sensitive *B. garinii* is mediated by the activation of the classical pathway given that *B. garinii* survives in C1q depleted human serum or anti-C1q treated serum (Dam et al., 1997). This observation suggests that differences between the serum sensitive *B. garinii* and the serum resistant *B. burgdorferi* strains may be useful in identifying proteins that provide serum resistant phenotypes to sensitive species.

Summary

The causative agent of Lyme disease, *B. burgdorferi*, is very different from most pathogenic bacteria both biologically and physiologically. One of the unique features of *B. burgdorferi* is the large number of lipoproteins that are produced on the bacterial surface in response to different environmental cues. The Rrp2-RpoN-RpoS regulatory pathway plays an important role for *B. burgdorferi* at the interface of tick vector and mammalian host (Boardman et al., 2008; Hubner et al., 2001; Radolf et al., 2012; Samuels, 2011) as this regulatory cascade up-regulates a large number of genes encoding lipoproteins such as *ospC*, *dbpBA*, and *bbk32*, which are important in mammalian infection (Grimm et al., 2004; Pal et al., 2004a;

Radolf et al., 2012; Samuels, 2011; Seshu et al., 2006; Weening et al., 2008). These surface lipoproteins are involved in various aspects of borrelial pathogenesis, including colonization, dissemination and immune suppression.

In addition to those lipoproteins, other uncharacterized borrelial lipoproteins are up-regulated in the mammalian host. Previous studies indicate that a putative lipoprotein, BBA33, is produced in an RpoS-dependent manner (Boardman et al., 2008; Caimano et al., 2007; Kumar et al., 2010). However, the significance and function for BBA33 was not identified. Given its linkage to the BosR/Rrp2-RpoN-RpoS regulatory cascade, we hypothesized that BBA33 facilitates *B. burgdorferi* infection in the mammalian host. In Chapter II, we report that the deletion of *bba33* eliminates *B. burgdorferi* infectivity in C3H mice, in a manner that could be rescued by genetic complementation with intact *bba33*. In regard to BBA33 function, a combinatorial peptide approach, coupled with subsequent *in vitro* binding assays, indicated that BBA33 binds to collagen type VI and, to a lesser extent, collagen type IV. Whole cell binding assays demonstrated BBA33-dependent binding to human collagen type VI. Taken together, these results suggest that BBA33 interacts with collagenous structures and may function as an adhesin in a process that is required to establish *B. burgdorferi* colonization. The striking phenotype of the *bba33* mutant in mice led us to ask if BBA33 interferes with the innate immune response by interacting with immune-response related proteins that may share a structure similar to collagens. C1q, the first protein involved in the activation of the classical complement

pathway, contains a collagen-like triple helical domain that we hypothesized might be recognized by BBA33. As such, we were interested in determining whether BBA33 interacts with C1q and asked if BBA33 was capable of inhibiting the activation of the classical complement pathway.

The human complement system is a connected network of blood proteins capable of recognizing and eliminating microbial intruders. *B. burgdorferi sensu lato* genospecies (i.e., *B. burgdorferi*, *B. afzelii*, and *B. garinii*) differ in their ability to survive in the presence of human complement (Breitner-Ruddock et al., 1997; Dam et al., 1997). Complement killing of serum sensitive *B. garinii* is mediated by the activation of the classical pathway (Dam et al., 1997), indicating that evasion of the classical pathway may be the key for serum-resistant *Borrelia* species to survive in human blood. However, the mechanisms of how *B. burgdorferi* evades the activation of classical pathway are still not well-understood. To answer this question, we tested if BBA33 inhibited the activation of the classical pathway. In a classical pathway dependent hemolysis assay, we found that BBA33 did not affect complement activation. However, surprisingly, we found that another *B. burgdorferi* surface exposed lipoprotein, BBK32, showed a very potent inhibitory effect on classical pathway activation.

In Chapter III, we investigated further the mechanism of how *B. burgdorferi* BBK32 blocks the activation of the classical complement pathway. We found that BBK32, a previously identified fibronectin-binding protein, targets and inhibits the first component of complement, C1 (Garcia et al., 2016). Upon binding to human

C1, BBK32 traps the C1 protease complex in an inactive state, and prevents the downstream proteolytic events of the pathway by direct binding to C1r. We further mapped this inhibitory function to the previously uncharacterized C-terminal domain of BBK32. This study defines a new mechanism by which microbes are able to escape the human innate immune system and identifies the complement protease C1r as a new target of bacterial complement inhibition. Thus, the discovery of the complement inhibitory activity of BBK32 may serve as a foundation to identify other related proteins from other *Borrelia* species and may serve as the basis for the development of therapeutically useful mimetics that mirror this BBK32-specific function (i.e, inhibit the activation of C1).

CHAPTER II

THE *Borrelia burgdorferi* BBA33 LIPOPROTEIN IS A COLLAGEN BINDING ADHESIN THAT IS REQUIRED FOR BORRELIAL PATHOGENESIS*

Introduction

The spirochetal bacterium *Borrelia burgdorferi*, the etiologic agent of Lyme disease, infects mammalian hosts via the bite of *Ixodes* spp. ticks (Radolf et al., 2012; Samuels, 2011). When humans become infected, they present with non-descript flu-like symptoms with most developing a characteristic skin lesion referred to as a erythema migrans (recently reviewed in (Shapiro, 2014)). If untreated, the infection can progress to a multi-stage disorder as the spirochete disseminates to distant organ sites, resulting in carditis, neurological disorders, and arthritis. Recently the CDC reported that more than 300,000 cases of Lyme disease are diagnosed annually in the United States, indicating that Lyme borreliosis is a significant, re-emerging infectious disease (Hinckley et al., 2014). Furthermore, the lack of an effective vaccine underscores the need to better understand factors that contribute to the pathogenic potential of *B. burgdorferi.*

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B. burgdorferi establishes infection by interaction with connective tissue of the skin and disseminates to deeper tissues following colonization (Cabello et al., 2007). Early in the infectious process, *B. burgdorferi* is known to align with host collagen (Barthold et al., 1991) and interact with other structures found within the extracellular matrix (ECM) in a process that involves the function of borrelial adhesins. Known surface exposed adhesins that recognize host structures found in the ECM include: the lipoproteins DbpA and DbpB that bind decorin and glycosoaminoglycans (GAG's) (Blevins et al., 2008; Fischer et al., 2003; Guo et al., 1995; Weening et al., 2008); BBK32 that interacts with fibronectin and GAG's (Fischer et al., 2006; Kim et al., 2004; Norman et al., 2008; Probert and Johnson, 1998; Seshu et al., 2006); BmpA and ErpX that engage laminin (Brissette et al., 2009a; Verma et al., 2009); P66 and BBB07 that adhere to integrin proteins (Behera et al., 2008; Ristow et al., 2012); and CspA and CspZ that bind to complement proteins, fibronectin, laminin, and collagens (Hallström et al., 2010, 2013; Kenedy et al., 2009; Rogers and Marconi, 2007). Several of these proteins are categorized as MSCRAMMs (for microbial surface component that recognize adhesive matrix molecules) (Patti et al., 1994) and some (Dbp's, BBK32, and P66) are linked to borrelial pathogenesis (Lin et al., 2012; Ristow et al., 2012; Seshu et al., 2006; Weening et al., 2008).

It is well established that *B. burgdorferi* modulates its gene expression as it cycles between the tick vector and the mammalian hosts they infect (Akins et al., 1998; Brooks et al., 2003; Liang et al., 2002; Narasimhan et al., 2003; Ohnishi et

al., 2001; Ojaimi et al., 2003; Revel et al., 2002; Samuels, 2011), including genes that encode infection-associated lipoproteins (Grimm et al., 2004; Pal et al., 2004a; Radolf et al., 2012; Seshu et al., 2006; Weening et al., 2008; Zhang et al., 1997). For mammalian infection, the Rrp2-RpoN-RpoS regulatory pathway plays an important role in this process (Boardman et al., 2008; Hubner et al., 2001; Radolf et al., 2012; Samuels, 2011) as this regulatory cascade is required for the expression of *ospC*, *dbpBA*, and *bbk32*, all of which are required for optimal mammalian infection (Grimm et al., 2004; Pal et al., 2004a; Radolf et al., 2012; Samuels, 2011; Seshu et al., 2006; Weening et al., 2008). In addition to these pathogenesis-associated genes, several additional genes are regulated in a similar manner; however, the importance and function of the proteins they encode are not known. One such gene that is RpoS-dependent is *bba33*, which is found on the 54 kb plasmid of *B. burgdorferi* strain B31 (Fraser et al., 1997). Previous studies showed that *bba33* was up-regulated 29-fold in implanted dialysis membrane chambers and 8.2-fold *in vitro* in an RpoS-dependent manner (Caimano et al., 2007), and required Rrp2 for maximal expression (Boardman et al., 2008), suggesting that BBA33 is produced under mammalian-like conditions. In support of this contention, Kumar *et al*. found that *bba33* is induced during a blood meal in ticks and is expressed in infected mouse skin (Kumar et al., 2010). Furthermore, microarray and RT-PCR experiments revealed that *bba33* transcripts were significantly up-regulated in heart tissue, but poorly expressed in the central nervous system in *B. burgdorferi* infected non-human primates (Narasimhan et al.,

2003), suggesting that *bba33* expression is spatially regulated and thus may contribute to the dissemination and secondary colonization of *B. burgdorferi* to targeted organs. Taken together, these data suggested that BBA33 was linked to *B. burgdorferi* virulence; however, its function was unknown.

By analogy to other infection-associated lipoprotein-encoding genes that are regulated in a similar manner (e.g., *dbpA* or *bbk32* (Blevins et al., 2008; Seshu et al., 2006; Weening et al., 2008)), we hypothesized that the protein encoded by *bba33* could contribute to pathogenesis-associated functions. We now report that the loss of *bba33* renders *B. burgdorferi* essentially non-infectious. A combinatorial peptide approach suggested that BBA33 recognizes a sequence that maps to human collagen type VI. Given its abundance in the skin and other sites colonized by *B. burgdorferi*, as well as the known association of *B. burgdorferi* with collagen (Barthold et al., 1991), we evaluated the ability of BBA33 to bind collagen and mediate bacterial attachment to collagen substrates. The results presented herein demonstrate that BBA33 plays an important role in early stages of borrelial infection due presumably to its ability to interact with collagenous substrates.

Results

Molecular analysis of bba33

Given the significance of several RpoS-regulated *B. burgdorferi* genes in mammalian infection (Blevins et al., 2008; Hubner et al., 2001; Pal et al., 2004a; Radolf et al., 2012; Weening et al., 2008), including several that map to the 54 kb

plasmid (lp54) of *B. burgdorferi* strain B31 (Blevins et al., 2008; Gilmore et al., 2010; Kumar et al., 2010; Weening et al., 2008), we sought to determine whether RpoS-dependent BBA33 contributed to borrelial pathogenesis. As a first step, we genetically deleted *B. burgdorferi bba33* as depicted in **Fig. 2A**. Briefly, a construct that replaced the entire *bba33* locus with a streptomycin resistant (Str^R) cassette was made and designated pHZ001. This construct was transformed into strain ML23 and transformants were screened by PCR for allelic exchange of the native *bba33* gene with the Str^R marker (**Fig. 2B**). ML23 is a strain that lacks the 25 kb linear plasmid that encodes a restriction/modification gene (*bbe02*); as such, this strain is more readily transformable via allelic exchange (Seshu et al., 2006; Weening et al., 2008), but is also non-infectious since the *bbe22*/*pncA* locus is essential for survival following experimental infection (Labandeira-Rey and Skare, 2001; Purser and Norris, 2000; Purser et al., 2003). Infectivity can be restored via trans complementation of *bbe22*/*pncA* on the shuttle vector pBBE22 (Purser et al., 2003).

To assess the status of the Str^R transformants relative the parent strain, PCR was employed. Consistent with the deletion of *bba33*, a 1296 bp PCR product was amplified from the putative Δ*bba33* strain using primers 1 and 4, relative to the expected 841 bp fragment from the parent strain (**Fig. 2B**). As predicted, primers 4 and 5 failed to amplify any fragment from the parent strain ML23 pBBE22*luc* whereas a Str^R-cassette containing 995 bp fragment was amplified from the putative Δ*bba33* mutant (**Fig. 2B**). Furthermore, primers 2 and

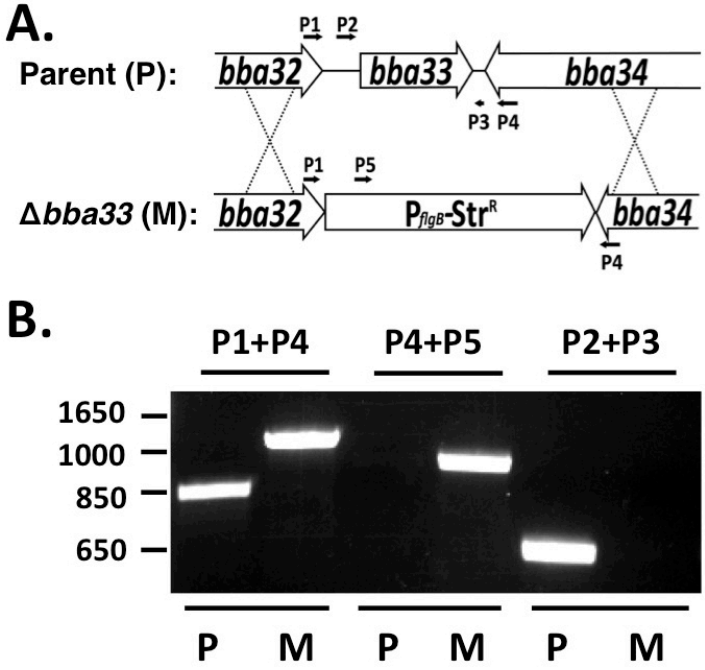

Figure 2. A schematic representation of the *bba33* deletion strategy. A. The *bba33* locus and its flanking region was replaced by a P_{flgB}-Str^R cassette by homologous recombination. B. Primer pairs P1/P4 and P4/P5 (Table 3) were used to confirm the presence of P_{flgB}-Str^R cassette in the *bba33* deletion mutant strain HZ001 (M, Δ*bba33* mutant) relative to its parent strain ML23 (P, parent) by PCR. Likewise, PCR with the primer pair P2/P3 (Table 3) confirmed that HZ001 carried the Δ*bba33*::Str^R allele (M).

3 amplified a 672 bp fragment from the parent, but not from the putative ∆*bba33* mutant (**Fig. 2B**). The resulting ∆*bba33* mutant strain was designated HZ001 and then transformed with pBBE22*luc* to both complement mammalian infectivity with *bbe22*, which is necessary in strains lacking lp25 (Purser et al., 2003), and provide a constitutively expressed borrelial codon optimized luciferase (*luc*) gene (Blevins et al., 2007).

To genetically complement the *bba33* deletion *in trans*, intact *bba33* with both upstream (210 bp) and downstream (129 bp) sequences included, was cloned into pBBE22*luc* (Hyde *et al.*, 2011). The resulting construct, designated pHZ300, was transformed into the *bba33* deletion strain HZ001. PCR analysis of the transformed deletion strain (**Fig. 3B**) showed a 672 bp fragment similar to that seen for the initial parent strain (**Fig. 2B**). *B. burgdorferi* plasmid profiles of all strains were evaluated to ensure no further loss of plasmid DNA (Labandeira-Rey and Skare, 2001) (data not shown). All three strains made PncA/BBE22 protein and produced light, consistent with the presence of the *pncA* locus and firefly *luc* on pBBE22*luc*, respectively (data not shown). Furthermore, all isolated strains grew at the same rate indicating that the loss of *bba33* did not alter *B. burgdorferi* replication *in vitro* (**Fig. 4**).

Conditions that mimic the mammalian host environment induce bba33

To determine whether BBA33 was produced in the deletion strain HZ001 pBBE22*luc* relative to its parent (ML23 pBBE22*luc*) and genetic complement

(HZ001 pHZ300), we subjected protein lysates to Western immunoblot analysis. Prior studies indicated that temperature, pH, and CO_2 content affected gene expression in *B. burgdorferi* (Carroll et al., 1999; Hyde et al., 2007; Samuels, 2011; Stevenson et al., 1995). Furthermore, *bba33* was induced under conditions that result in the production of several virulence-associated proteins, including OspC, BBK32, and DbpA, which require Rrp2/RpoS for their induction (Grimm et al., 2004; Pal et al., 2004a; Seshu et al., 2006; Weening et al., 2008). To assess whether the aforementioned variables contributed to *bba33* induction, we subjected the aforementioned three strains to growth at either 32˚C, 1% CO_2, pH 7.6 (our conventional growth conditions) or 37˚C, 5% CO_2, pH 6.8 to mimic the mammalian host conditions *in vitro*. When equivalent amounts of *B. burgdorferi* proteins from these conditions were resolved by SDS-PAGE, lp54-encoded BBA33 was detected in the parent strain ML23 pBBE22*luc* grown at 37˚C, 5% CO_2, pH 6.8 (**Fig. 5**, bottom panel, right), consistent with the induction of mammalian-induced proteins, including OspC (**Fig. 5**, middle panel). In contrast, lp54-encoded BBA33 was not detectable in ML23 pBBE22*luc* when grown at 32˚C, 1% CO_2, pH 7.6 (**Fig. 5**, bottom panel, left). In fact, BBA33 levels were only observed at 37˚C, 5% CO_2, pH 6.8; no other combination of temperature, CO_2 content, or pH resulted in detectable levels of BBA33 (data not shown). As expected, BBA33 was not detected in the ∆*bba33* mutant HZ001 pBBE22*luc*, regardless of growth condition (**Fig. 5**, lower panel). In contrast, when *bba33* was expressed from a shuttle vector in the genetic complement strain HZ001 pHZ300

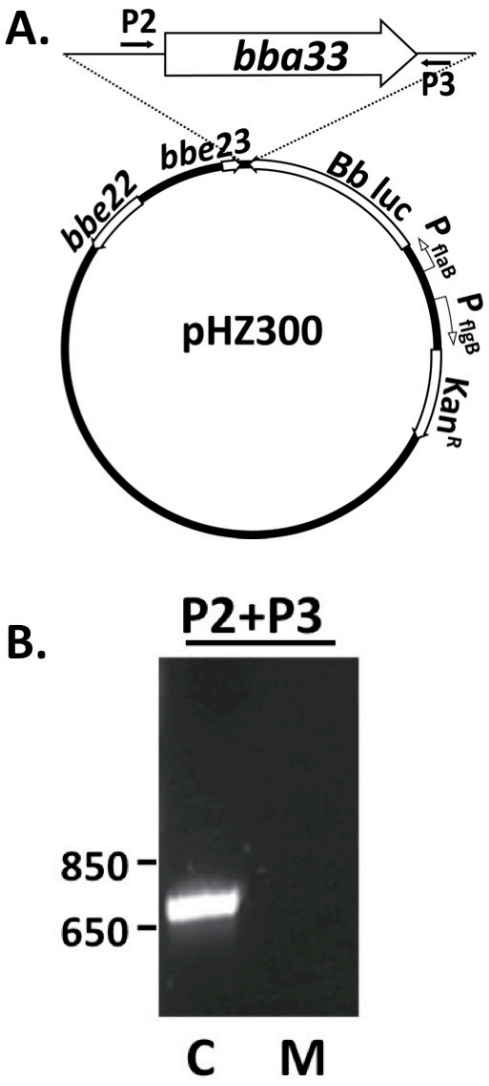

Figure 3. A schematic presentation of the *bba33* complementation strategy. A. Schematic presentation of the *bba33* complementation construct pHZ300B. PCR using primer pairs P2/P3 confirmed the presence of *bba33* in the complemented strain HZ001 pHZ300 (C; complement) relative to the Δ*bba33* strain HZ001 pBBE22*luc* (M; Δ*bba33* mutant).S

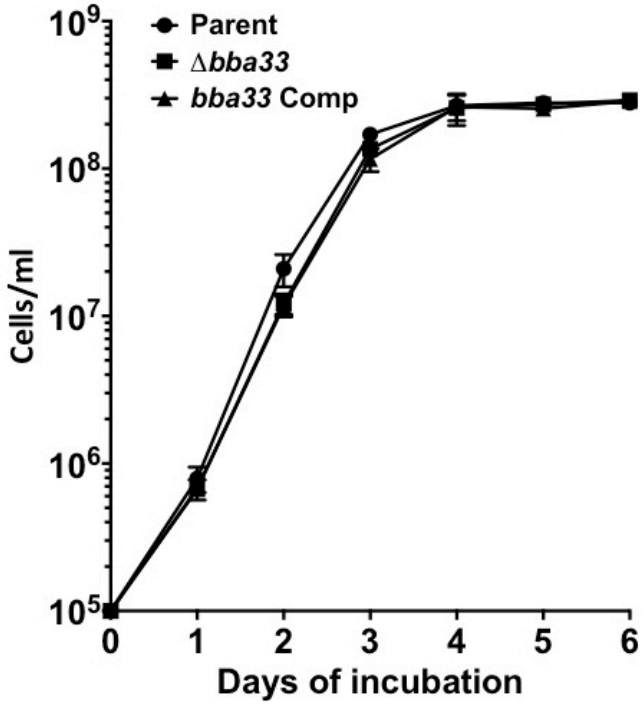

Figure 4. *In vitro* growth of the *B. burgdorferi* strains used in this study. The *B. burgdorferi* Parent strain (ML23/pBBE22*luc*), the Δ*bba33* mutant (HZ001/pBBE22*luc*), and the *bba*33 complemented strain (HZ001/pHZ300; *bba33* Comp.) were grown in triplicate in BSK-II media at 37˚C, 5% CO_2, pH 6.8 and enumerated by dark field microscopy daily out to day 6. Similar growth kinetics between these 3 strains were observed when the cells were grown at 32˚C, 1% CO_2, pH 6.8, as well as other growth conditions (not shown). Data points shown reflect average value with standard error. No significant differences in growth were observed.

(**Fig. 5**, lower panel), the levels of BBA33 produced were high independent of the conditions imposed, presumably due to the increased copy number of the episomal DNA relative to its native location on lp54, which may titrate out regulatory components that regulate *bba33* expression. In support of this premise, previous studies estimated the copy number of cp9 at 5-10 per cell relative to linear replicons (Beaurepaire and Chaconas, 2007; Tilly et al., 2006). In addition, a similar effect was seen when *dbpBA* was complemented *in trans*; that is, shuttle-vector encoded DbpA was produced at levels that were approximately 8-fold greater than the lp54 encoded version (Weening et al., 2008). Given that *dbpBA* and *bba33* are similarly regulated (Blevins et al., 2008; Weening et al., 2008), it is not surprising that *in trans* encoded BBA33 is produced at a similarly high level.

BBA33 is a surface exposed lipoprotein

The primary sequence of *B. burgdorferi* BBA33 predicts a lipoprotein with a leader peptidase II signal peptide at its amino terminus. To provide supporting evidence that BBA33 was surface exposed, we subjected intact *B. burgdorferi* strain ML23 pBBE22*luc* to proteinase K treatment. These cells were grown at 37˚C, 5% CO_2, pH 6.8, to induce detectable levels of BBA33 (**Fig. 5**). The data showed that BBA33 was degraded in intact *B. burgdorferi* cells under conditions where P66 was accessible to protease (producing a known 50 kDa stable product; (Bunikis et al., 1998)) but subsurface endoflagella (FlaB) was not (**Fig. 6**). The addition of detergent Triton X-100 together with proteinase K resulted in the loss

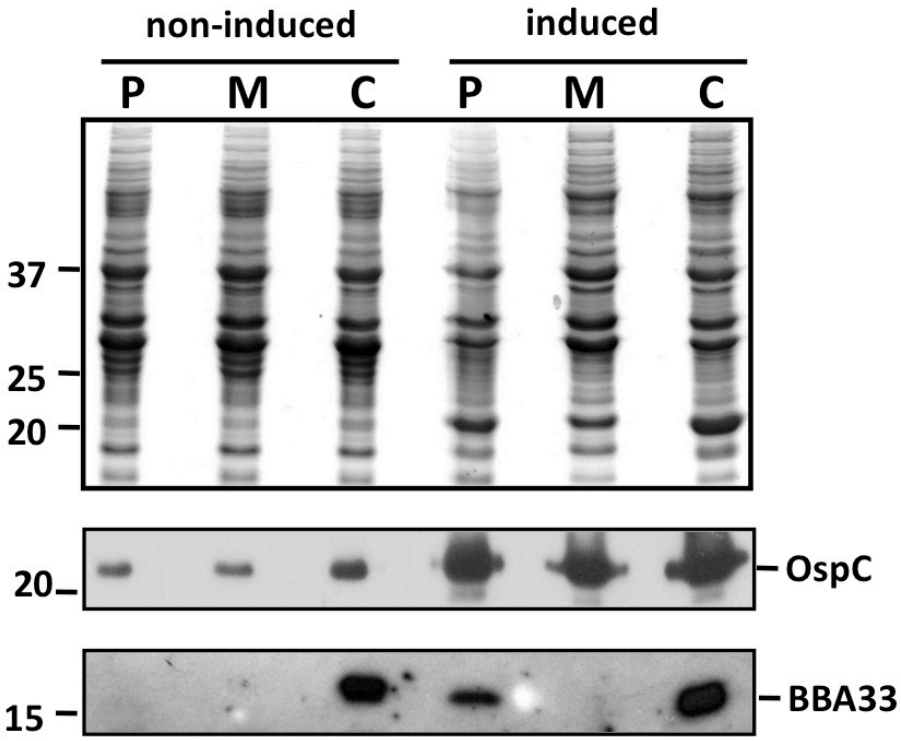

Figure 5. BBA33 is produced under conditions that mimic the mammalian-like environment. Equivalent amount of protein were loaded for the *B. burgdorferi* parent strain ML23 pBBE22*luc* (P for parent), the Δ*bba33* strain HZ001 pBBE22*luc* (M for mutant), and the genetic complement HZ001 pHZ300 (C for complement) from cells that were either grown in media under "non-induced" (32˚C, 1% CO_2, pH 7.6; left 3 lanes) or "induced" conditions (37˚C, 5% CO_2, pH 6.8; right 3 lanes), separated by SDS-PAGE, and stained with Coomassie blue (top panel) or immunoblotted with anti-OspC (middle panel), or anti-BBA33 serum (bottom panel). Note that the only condition where the parent strain made BBA33 is shown; no other combination of temperature, CO_2 content, or pH resulted in the detection of BBA33. The numbers on the side panel depict the molecular mass of protein markers (in kDa).

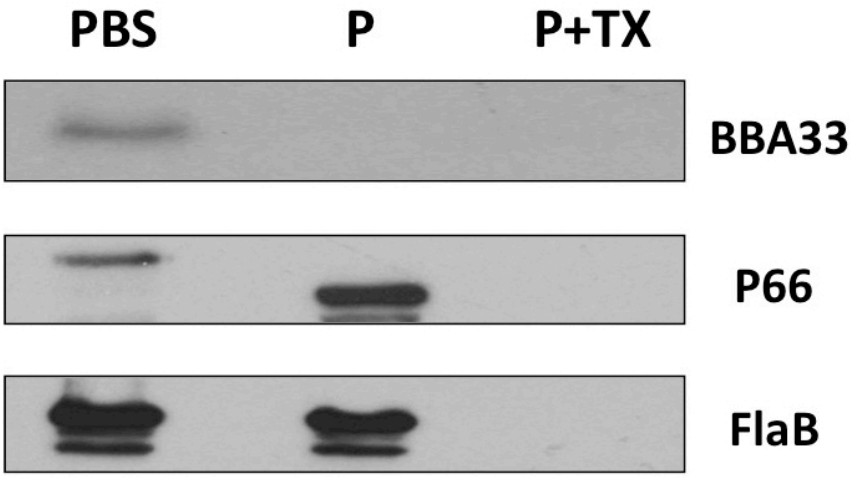

Figure 6. BBA33 is surface exposed. *B. burgdorferi* strain ML23 pBBE22*luc* was cultivated at 37˚C, 5% CO_2, pH 6.8 (inducing conditions) and harvested. The cells were then either resuspended in buffer (PBS), incubated with Proteinase K (P), or treated with both Proteinase K and Triton X-100 (P+TX). Following processing, the resulting samples were subjected to SDS-PAGE and immunoblotted with antiserum directed against either BBA33, the outer membrane P66 protein, or the subsurface FlaB protein.

of all antigens tested providing further support that the cells treated with PBS or proteinase K alone were intact (**Fig. 6**). Additional studies confirmed that BBA33 partitioned with Triton X-114, consistent with its predicted status as a lipoprotein (not shown). Taken together, these results demonstrate that BBA33 is a surface exposed outer membrane lipoprotein of *B. burgdorferi*.

The loss of bba33 results in the rapid clearance of B. burgdorferi

Next, we asked if *B. burgdorferi* cells lacking *bba33* were attenuated using the murine infection model. To track the active infection of cells lacking BBA33, we infected C3H/HeN mice with the parent strain (ML23 pBBE22*luc*), the ∆*bba33* strain (HZ001 pBBE22*luc*), and the *bba33* complement strain (HZ001 pHZ300), all containing borrelial codon optimized firefly luciferase at doses of 10^3 and 10^5 cells and measured light emission from these cells over time (**Fig. 7 and 8**). At the lower inoculum dose, a signal is not detected above background levels for any of the strains prior to day 4. At day 4 a clear signal is observed in mice infected with the parent and complement strain (**Fig. 7**). The signal expanded with a peak at day 7 and then began to wane commensurate with the development of the adaptive immune response (**Fig. 7 and 8**). At the low dose, the ∆*bba33* mutant did not emit light above background for any of the time points tested (**Fig. 7**). For the high dose, an equivalent light signal was detected at 24 hours following infection for all strains tested (**Fig. 7 and 8**). However, at day 4 and beyond, no signal above background was detected in the ∆*bba33* strain HZ001 pBBE22*luc*

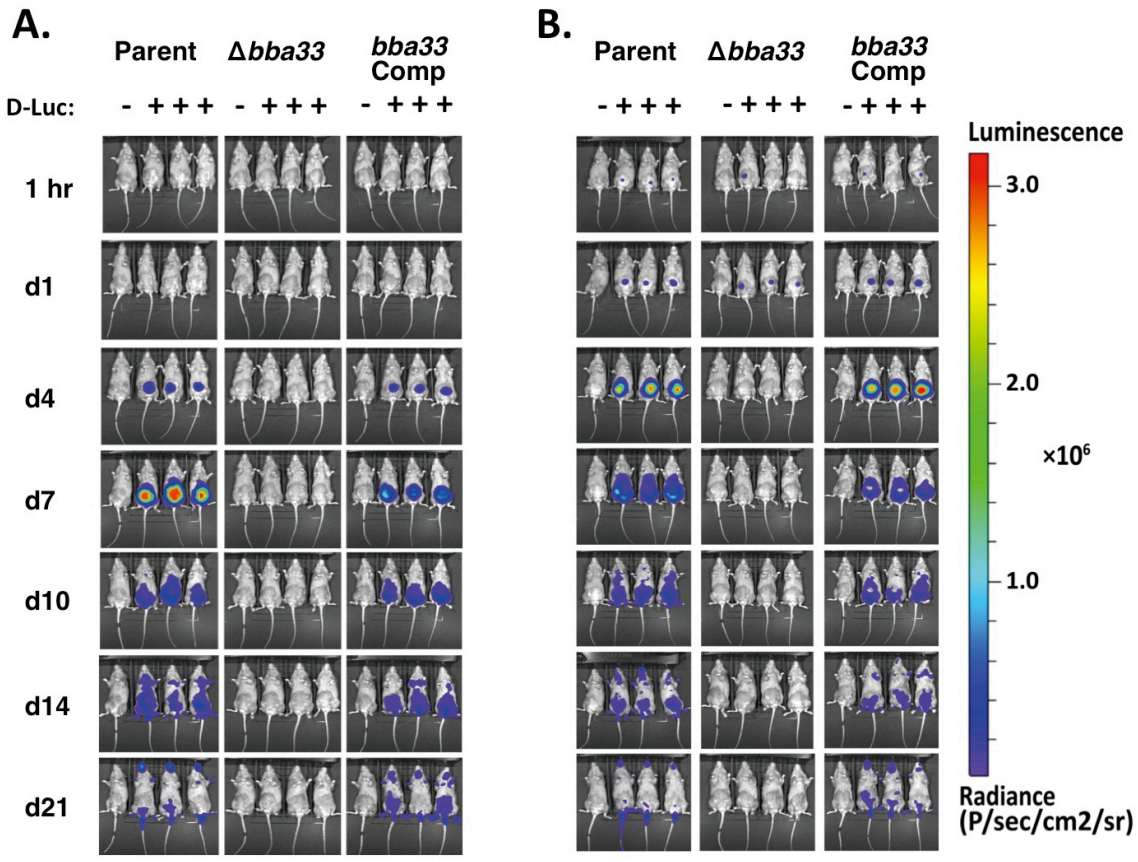

Figure 7. Temporal and spatial tracking of *B. burgdorferi* strains following infection with 10^3 and 10^5 spirochetes. C3H mice were infected with the parent (Parent; ML23/pBBE22*luc*), the Δ*bba33* mutant (Δ*bba33*; HZ001/pBBE22*luc*), or the *bba33* complemented strain (*bba33* Com., HZ001/pHZ300) at a dose of 10^3 (A) and 10^5 (B). Mice denoted with "+" were treated with D-luciferin and imaged at the times listed on the left. For each image shown the mouse on the far left (denoted as "-") was infected with *B. burgdorferi* but did not receive D-luciferin to serve as a background control. All images were normalized to the same scale (shown on the right).

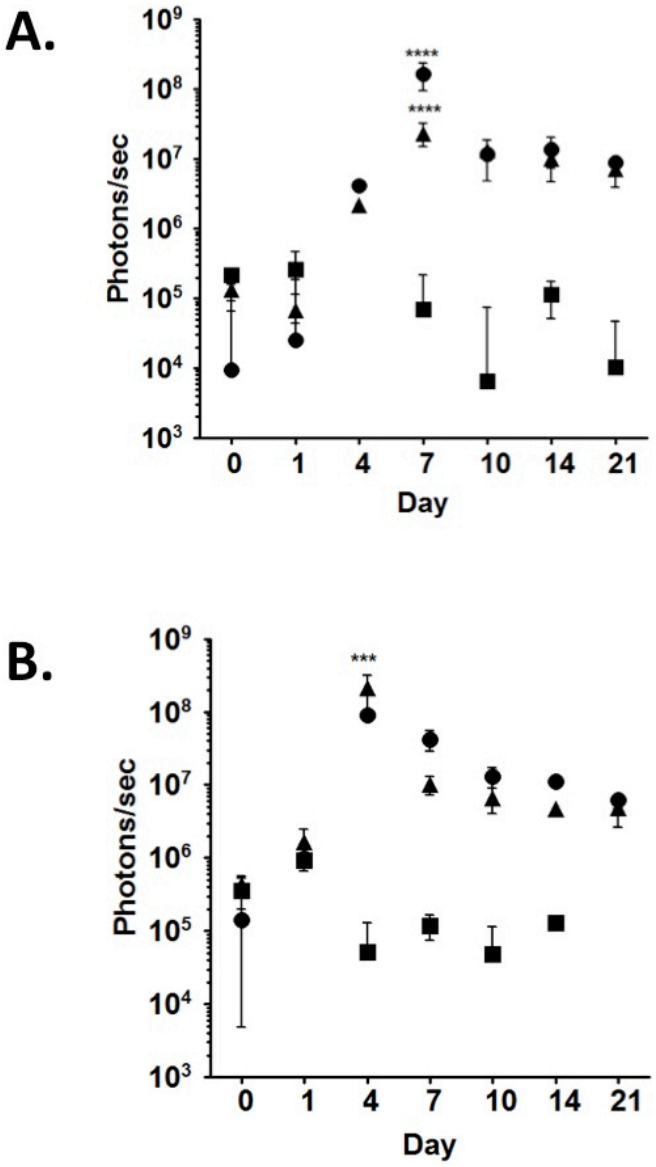

Figure 8. Quantification of *in vivo* luminescence images of mice infected at a dose of 10^3 (A) and 10^5 (B) as previously described. *B. burgdorferi* ML23/pBBE22*luc* is depicted as circles, the Δ*bba33* derivative HZ001/pBBE22*luc* as squares, and the genetic complement HZ001/pHZ300 as triangles. Each time point represents the average value from the three mice given luciferin. ***$P < 0.001$; **** $P < 0.0001$.

(**Fig. 7**). Conversely, the parent and *bba33* complement strains exhibited similar light emission and dissemination profiles (**Fig. 7 and 8**). After 21 days, the mice infected with the parent, ∆bba33, and bba33 complement, at both inoculum doses, were sacrificed and organs were harvested for cultivation and quantitative PCR analysis. Consistent with the imaging data, the absence of bba33 resulted in the inability of B. burgorferi to survive or colonize mice from any organ site tested at any inoculum dose tested including up to an infection dose of 107 ∆bba33 B. burgdorferi (Table 1). This is in stark contrast to the parent and complement strains, which were recovered from all organs independent of dose (Table 1).

To quantify the bacterial burden, quantitative PCR (qPCR) was performed on total DNA extracted from the skin, lymph node, tibiotarsal joint, and heart from mice infected with the parent, the ∆*bba33* mutant, and *bba33* complemented strains harvested at the same time as the *in vitro* cultivated samples. Consistent with the cultivation data shown in **Table 1**, few genomic equivalents were detected in tissues infected with the ∆*bba33* mutant HZ001 pBBE22*luc* at a 10^3 or 10^5 inoculum (**Fig. 9A and 9B**, respectively). Also in line with the prior infection data (**Fig. 7 and Table 1**), the parent and complemented strains exhibited similar colonization phenotypes indicating that the defect observed in the mutant was due to the loss of BBA33 and not a secondary mutation (**Fig. 9A and 9B**). Interestingly, the overproduction of BBA33 in complemented strains resulted in lower colonization in joint tissue relative to the parent strain independent of

Table 1. Infectivity of the Δ*bba33* mutant strain relative to its parent and genetic complement.

		Number of culture positive/total number						
Strain	Inoculum dose	Lymph node	Skin	Heart	Spleen	Bladder	Joint	All sites
ML23/pBBE22*luc*	10^3	4/4	4/4	4/4	4/4	4/4	4/4	24/24
(parent)	10^5	4/4	4/4	4/4	4/4	4/4	4/4	24/24
HZ001/pBBE22*luc*	10^3	0/4	0/4	0/4	0/4	0/4	0/4	0/24
(Δ*bba33*)	10^5	0/4	0/4	0/4	0/4	0/4	0/4	0/24
	10^7	0/3	0/3	0/3	0/3	0/3	0/3	0/18
HZ001/pHz300	10^3	4/4	4/4	4/4	4/4	4/4	4/4	24/24
(Δ*bba33/bba33*	10^5	4/4	4/4	4/4	4/4	4/4	4/4	24/24
Comp)								

infectious dose, yet exhibited similar bacterial burden in heart tissue (**Fig. 9A and 9B**). Taken together, these data indicate that BBA33 is required for infectivity. Furthermore, the absence of BBA33 from the surface of *B. burgdorferi* leads to rapid clearance, suggesting that BBA33 is required for the establishment of a localized infection and resistance to the host innate immune response.

Recombinant BBA33 mediates binding to collagens

Given that BBA33 was an infectivity-associated surface exposed lipoprotein, we were next interested in identifying a function for this protein. To this end, we screened random cyclic peptide phage display libraries to identify peptide sequences that would selectively recognize recombinant BBA33 (rBBA33) (Barbu et al., 2010; Mullen et al., 2006; Sergeeva et al., 2006). We found that the number of phage binding to immobilized rBBA33 increased relative to background after several rounds of panning (based on BSA binding), suggesting a specific enrichment for BBA33. After the aforementioned panning, the DNA corresponding to the peptide insert from 100 randomly selected plaques was sequenced. An alignment of the deduced amino acids revealed enrichment for the sequence PGEPGLN, which is found in the human collagen type VI alpha 3 chain. However, in collagen this sequence is present in the triple helix collagenous domains and it is unlikely that all residues identified in the cyclic peptide would be available for interactions in the structurally restricted triple helix. To explore the possibility that BBA33 can bind collagens we used an ELISA-type

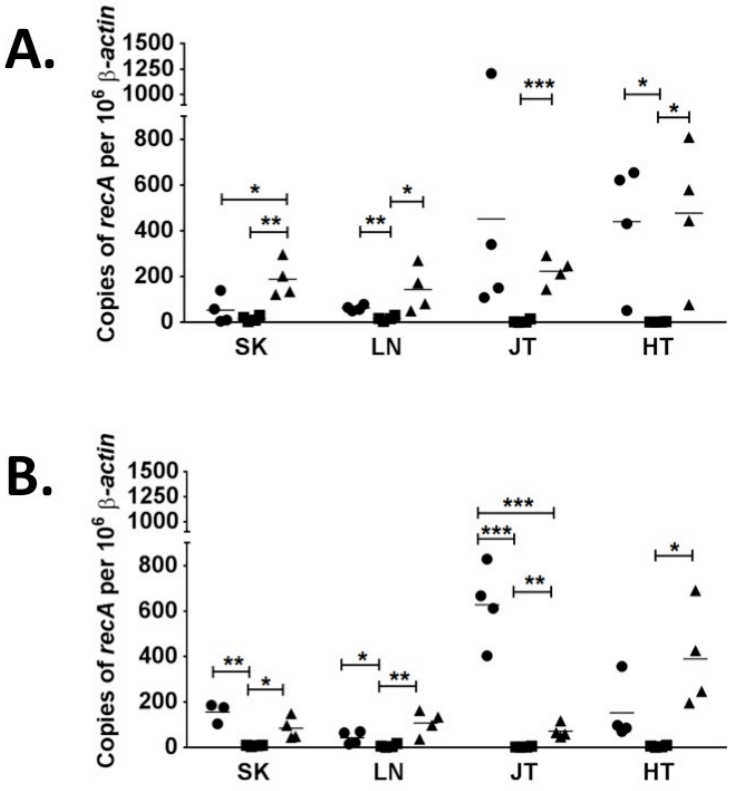

Figure 9. Quantitative assessment of *B. burgdorferi* infection in C3H mice. Real-time PCR (qPCR) of the parental strain *B. burgdorferi* ML23/pBBE22*luc* (Circles), the Δ*bba33* derivative HZ001/pBBE22*luc* (Squares), and the genetic complement HZ001/pHZ300 (Triangles) was used to enumerate borrelial genomic equivalents from mouse tissues. Mice were infected with either 10^3 (A) or 10^5 (B) *B. burgdorferi* strains for 21 days before total DNA was obtained from skin (SK), lymph nodes (LN), joints (JT) and heart (HT) tissues. The results are represented as the number of borrelial genome copies per 10^6 mouse β-actin copies. The horizontal line depicts the mean value. Each data point shown represents an independent sample from a single mouse tissue assayed in triplicate and averaged. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

binding assay where increasing concentrations of purified rBBA33 protein were incubated in wells coated with different collagen types and other extracellular matrix proteins. A concentration dependent binding of BBA33 to type VI collagen was noted with saturation at 2 μM and half maximum binding (e.g., apparent K_D) at approximately 350 nM (**Fig 10A**). In contrast, purified OspC did not bind to collagen type VI at any concentration tested (**Fig. 10A**) indicating that the BBA33::collagen type VI interaction is specific. BBA33 showed dose-dependent binding to collagen type IV but not to human collagen type I, fibronectin, and murine laminin, indicating specificity for a subset of targets (**Fig 10B**). When BBA33 was subjected to heat inactivation, the binding to collagen observed was eliminated suggesting that the native conformation was required for dose-dependent binding (not shown).

Native BBA33 binds to type VI collagen

We next sought to determine whether native BBA33, presented on the surface of *B. burgdorferi* cells, could promote bacterial attachment to the identified BBA33 targets. A significant difference in borrelial cell attachment was observed to collagen type VI when the *bba33* complement (HZ001 pHZ300; designated C) was compared to the parent strain ML23 pBBE22*luc* (designated P) and Δ*bba33* strain HZ001 pBBE22*luc* (designated M) when the cells are grown at 32˚C, 1% CO_2, pH 7.6 (**Fig. 11, left**); under these conditions no difference was seen in the attachment to laminin to any of the strains tested (**Fig. 11**). Under these conditions

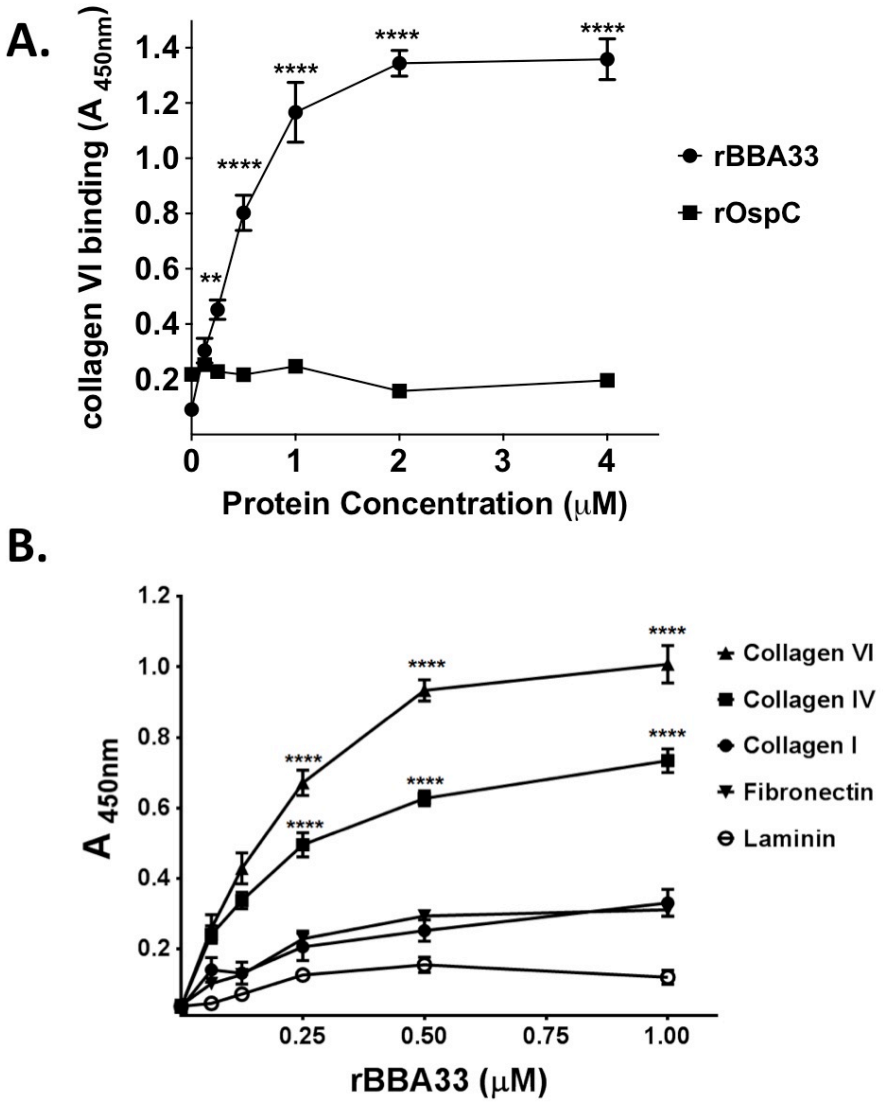

Figure 10. BBA33 binds to human type VI collagen and mouse type IV collagen in a dose-dependent manner. A. Various concentrations of purified BBA33 were incubated with type VI collagen. Identical concentrations of OspC were tested for type VI collagen interaction as well and served as a negative control. B. Binding of BBA33 to collagens types I, IV, and VI, as well as fibronectin and laminin, was evaluated as in panel A. Binding to collagens type VI and IV was dose dependent, whereas binding to fibronectin and laminin did not demonstrate specific binding to BBA33. All assays were done independently three times, each time in duplicate. $^{**}P < 0.01$; $^{****}P < 0.0001$.

the levels of BBA33 are low in the parent strain (**Fig. 5**); therefore, the low level of binding observed for the parent strain ML23 pBBE22*luc* is consistent with the lack of BBA33 produced when the parent is grown under non-inducing conditions and thus, not surprisingly, comparable to the Δ*bba33* mutant (**Fig. 11, left**). However, when the cells were grown under conditions that promote the production of BBA33 (**Fig. 5**; e.g., 37˚C, 5% CO_2, pH 6.8), a significant difference in collagen type VI binding was observed between the parent ML23 pBBE22*luc* and the Δ*bba33* mutant HZ001 pBBE22*luc*. No significant differences to laminin binding were noted (**Fig. 11**, right). As expected, the *bba33* complement HZ001 pHZ300 strain bound collagen type VI under inducing conditions (**Fig. 11**, right) since shuttle vector encoded BBA33 is synthesized at high levels independent of growth parameters (**Fig. 5**). These data are consistent with the induction of *bba33* under the "mammalian-like" induction conditions and provide further support that surface-exposed, native BBA33 specifically recognizes collagen type VI.

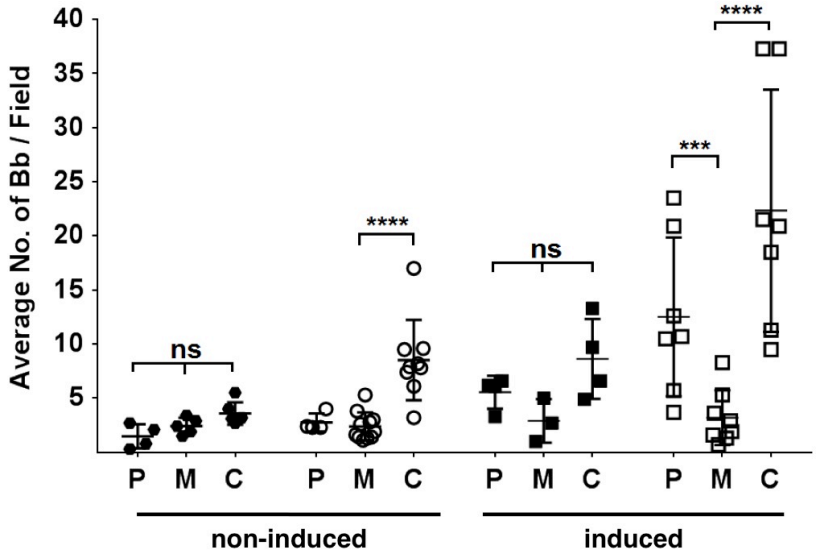

Figure 11. *B. burgdorferi* whole cells that produce surface exposed BBA33 mediate specific binding to human type VI collagen. *B. burgdorferi* strain ML23/pBBE22*luc* (P; parent), HZ001/pBBE22*luc* (M; *bba33* mutant), and HZ001/pHZ300 (C; complement) were grown either under "non-inducing" conditions (32˚C, 1% CO_2, pH 7.6; left side) or "inducing" conditions (37˚C, 5% CO_2, pH 6.8; right side) and incubated on cover slips containing either mouse laminin (filled circles or squares) or human type VI collagen (open circles or squares). *B. burgdorferi* cells binding was scored via dark field microscopy. Each data point represents the average number of spirochetes visualized in 10 random fields. Each strain was tested a minimum of 3 times, with each sample assayed in duplicate. ***$P < 0.001$; ****$P < 0.0001$; ns, non-significant difference.

Discussion

Interaction with the host extracellular matrix (ECM) is an essential part of the pathogenic process of *B. burgdorferi*. *B. burgdorferi* is transmitted from ticks to a mammalian host during a blood meal and, as a result, is deposited within the dermal layer of the skin where collagen and other components of the ECM reside (Cabello et al., 2007; Radolf et al., 2012). Some of the first micrographs of *B. burgdorferi* from infected tissue showed spirochetal bacteria aligned with collagen fibrils (Cabello et al., 2007; Barthold et al., 1991). Subsequent infectivity studies demonstrated that the association of *B. burgdorferi* with ECM tissue is required for persistence (Brissette and Gaultney, 2014; Cabello et al., 2007), although the basis for all *B. burgdorferi*-ECM interactions was not completely defined.

In this study we describe a collagen binding lipoprotein from *B. burgdorferi*, designated BBA33, which is required for experimental Lyme borreliosis. To date, the only data associated with *bba33* is its dependence on the BosR/Rrp2-RpoN-RpoS regulatory pathways for expression (Caimano et al., 2007). Despite the observation that *bba33* was subject to the same degree of regulation observed for other borrelial virulence determinants (Boardman et al., 2008; Caimano et al., 2007; He et al., 2007; Hubner et al., 2001; Yang et al., 2003), notably *ospC*, *dbpBA,* and *bbk32* (He et al., 2007; Hubner et al., 2001), no function was known for BBA33. Using phage display, we identified a sequence found in type VI collagen as a target for BBA33. To biochemically validate the predicted BBA33-collagen interaction we used an ELISA-based assay and found that BBA33 binds

to collagen types IV and VI in a dose-dependent manner, but does not bind to collagen I, laminin, or fibronectin (**Fig. 10B**). To address the ability of surface exposed native BBA33 to recognize ECM proteins, we incubated whole cells producing BBA33 with human collagen type VI and laminin. The results in **Fig. 11** show that the genetic complement strain HZ001 pHZ300, which produces large amounts of BBA33, significantly enhances the ability of the spirochete to adhere to collagen type VI relative to the parent strain (ML23 pBBE22*luc*) and ∆*bba33* strain (HZ001 pBBE22*luc*) that are grown under conditions where BBA33 is not produced (**Fig. 5**). These data appears to contradict earlier findings that saw no binding of *B. burgdorferi* to collagens (Guo et al., 1995). However, upon reflection, this observation may be consistent with our findings since lp54-encoded *bba33* is only expressed in the parent strain under conditions that mimic mammalian host adapted conditions (**Fig. 5**). The prior work was done using conventionally grown *B. burgdorferi* (Guo et al., 1995), which represent experimental conditions that do not yield detectable levels of BBA33 (**Fig. 5**). In addition, when the parent strain ML23 pBBE22*luc* is grown under inducing conditions, the level of collagen type VI binding increases significantly consistent with the production of BBA33 within this same environment (**Fig. 5**).

The ability of bacteria to adhere to collagen type VI is known for a few pathogens, including *Legionella* and some *Staphylococcus* spp. (Bober et al., 2010; Liu et al., 2004; Wagner et al., 2007), but is still poorly characterized relative to other pathogen-collagen interactions (Liu et al., 2007; Zong et al., 2005). It is

important to note that, in the case of collagen type VI binding by the *Staphylococcus capitis* protein SdrX, the recombinant protein bound to several types of collagens in ELISA-based binding assays but only type VI collagen when *S. capitis* cells were used to query collagen binding (Liu et al., 2004). As such, the ability of *B. burgdorferi* cells to bind to collagen type VI provides similar support for BBA33-type VI collagen interactions.

The recognition of collagen type VI by BBA33 is of further interest given the recent reports demonstrating the ability of activated macrophages to secrete this particular collagen in a non-fibrillar form as is normally seen in fibroblasts (Schnoor et al., 2008). Two molecules that stimulate the production and secretion of type VI collagen include IL-10 and TGF-β1, both of which are induced early in *B. burgdorferi* infection (Codolo et al., 2008; Lazarus et al., 2008). In addition, both of these cytokines negate the inflammatory response of cells, including activated macrophages (Bogdan and Nathan, 1993; Oosting et al., 2014) and, by virtue of their ability to secrete collagens, notably type VI collagen (Schnoor et al., 2008), might create an additional platform for *B. burgdorferi* to bind. Based on the concurrent deactivation of previously activated macrophages, this microenvironment may promote localized colonization of *B. burgdorferi* and assist in the dissemination of spirochetes to distal sites. Based on our current data it is unclear if this is operative *in vivo*; however, the dramatic attenuation of cells lacking BBA33 suggests that this lipoprotein plays an important role early in *B. burgdorferi* infection.

Type VI collagen also interacts with both biglycan and decorin, two proteoglycans that *B. burgdorferi* recognizes via the surface exposed lipoproteins, DbpA and DbpB (Brown et al., 1999; Fischer et al., 2003). It is tempting to speculate that *B. burgdorferi* infection stimulates the production of type VI collagen locally within the skin via innate immune cells and fibroblasts to generate a scaffold that borrelial cells can actively engage via a number of adhesins that bind to ECM targets. Further experimentation is required to test this hypothesis.

Perhaps the more striking result presented herein is the absolute loss of infectivity seen for the ∆*bba33* mutant strain HZ001 pBBE22*luc*. From our imaging data of bioluminescent spirochetes (**Fig. 7**), it is clear that the ∆*bba33* mutant is cleared early in the infectious process at time points where the parent and genetic complement are replicating *in vivo*, suggesting that BBA33 may assist in the resistance to innate immune clearance. It is curious that the complement protein, C1q, contains a collagen-like motif (Kishore and Reid, 2000). Although C1q is involved in the classical pathway of complement, a serum sensitive *B. burgdorferi sensu lato* isolate*, B. garinii,* is resistant to serum when C1q is selectively depleted (Dam et al., 1997), suggesting that the interaction of C1q and *Borrelia* isolates is key for innate survival. Our evaluation of BBA33::C1q interactions indicated that BBA33 does recognize and bind strongly to C1q (not shown). When we tested whether BBA33 could inhibit classical complement activation, no inhibitory activity was observed. However, surprisingly (and serendipitously), we did find that the fibronectin-binding protein, BBK32, was able to inhibit the classical pathway at a

K_d value of approximately 20 nM, indicating a high affinity interaction. This interaction serves as the basis for the next section of this dissertation, i.e., Chapter III.

A diverse set of pathogens are known to bind to mammalian collagen-rich tissues to promote colonization (Bober et al., 2010; Foster et al., 2014; Umemoto et al., 1997). The structure of several of these interactions has been resolved yielding important insight into the binding process (Zong et al., 2005). The best characterized is the Cna collagen binding protein from *Staphylococcus aureus*, which uses a collagen-hug mechanism to bind host collagen (Zong et al., 2005). This interaction is important for pathogenesis, as Cna is required for several pathogenic features associated with experimental *S. aureus* infections, including ocular keratitis, osteomyelitis, and arthritis (Foster et al., 2014). The portion of Cna that mediates collagen binding is composed of two sub-domains, each adopting an IgG-like fold (Zong et al., 2005). This same beta pleated sheet rich structure is seen or presumed for the homologous collagen binding protein from *Streptococcus* and *Enterococcus* spp (Lannergard et al., 2003; Liu et al., 2007). Several algorithms predict that BBA33 is devoid of beta pleated sheet structure and instead is largely alpha helical in nature. If this is true, then the mechanisms that BBA33 employs to bind collagen are likely to be novel relative to that seen for Cna and related collagen binding proteins.

In summary, herein we demonstrate that *B. burgdorferi* BBA33 is a surface exposed lipoprotein that is expressed preferentially under conditions that mimic

the mammalian host environment. In addition, we show that BBA33 mediates specific interaction of *B. burgdorferi* with collagen substrates, i.e., human collagen type VI and murine type IV collagen. Despite a long history of *B. burgdorferi*-collagen interactions, to our knowledge BBA33 represents the first *B. burgdorferi* protein that recognizes human collagens in a dose-dependent manner. Imaging of bioluminescent ∆*bba33 B. burgdorferi* showed that the loss of BBA33 results in rapid clearance, which suggests a role in resistance to innate immune mechanisms. Further studies are warranted to investigate the significance of this interaction *in vivo*.

Experimental procedures

Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are described in **Table 2**. *Escherichia coli* strains were grown with aeration in Luria broth (LB) media at 37°C. Concentrations of antibiotics used in *E. coli* for selective pressure are as follows: kanamycin, 50 µg ml^{-1}; spectinomycin, 50 µg ml^{-1}. *B. burgdorferi* B31 ML23 (Labandeira-Rey and Skare, 2001) and derivative strains were grown in BSK-II media supplemented with 6% normal rabbit serum (Pel-Freez Biologicals, Rogers, AR) under either conventional microaerobic conditions at 32°C, at pH 6.8 or pH 7.6, under a 1% or 5% CO_2 atmosphere, or at 37°C, pH 6.8 or pH 7.6, under a 1% or 5% CO_2 atmosphere. For most experiments, *B. burgdorferi* cultures were grown at either 32°C, 1% CO_2, pH 7.6 (non-inducing or conventional conditions)

or at 37°C, 5% CO_2, pH 6.8 (inducing conditions). Growth was scored daily by dark field microscopy. Antibiotics used for the selective pressure in *B. burgdorferi* are as follows: kanamycin at 300 μg ml^{-1} and/or streptomycin at 50 μg ml^{-1}, depending on genetic composition. The use of infectious *B. burgdorferi* in this study was reviewed and approved by the Institutional Biosafety Committee at Texas A&M University.

Genetic modification of B. burgdorferi

B. burgdorferi deleted for *bba33* was generated via homologous recombination by replacing all of *bba33* with a P_{flgB}-*aadA* (Str^R) antibiotic cassette (Frank et al., 2003). To accomplish this, a 1526 bp fragment upstream of *bba33* was amplified using the primer pairs BamHI-A33us-recF and flgB-A33us-recR (**Table 3**) that created a 5' overhang to pCR2.1 with the *Bam*HI site and a 3' overhang homologous to P_{flgB} sequences from pKFSS1 (**Table 3**). An additional 1527 bp PCR product, which was directed to sequences downstream from *bba33*, was engineered with primers aadA-A33ds-recF and A33ds-XhoI-recR (**Table 3**) with overlapping sequences to sequences downstream to the stop codon of the *aadA* locus (Str^R) at the 5' end, and sequences homologous to pCR2.1, including the *Xho*I site. Finally, a PCR product containing the P_{flgB}-Str^R cassette was amplified from plasmid pKFSS1 using primer set FlgB-F and aadA-R (**Table 3**). All 3 PCR products, each containing homologous sequences, along with pCR2.1 digested with *Bam*HI and *Xho*I, were combined and subjected to Gibson

assembly as outlined by the manufacturer (New England Biolabs). The resulting construct was designated pHZ001 (**Table 3**). Transformation of strain ML23 with linearized pHZ001 was done as previously described (Samuels, 1995; Weening *et al.*, 2008; Hyde *et al.*, 2011). Transformants were selected for their resistance to streptomycin and screened by PCR to confirm the deletion of *bba33* using primers listed in **Table 3** and as depicted in **Fig. 2**. Candidates were screened for borrelial plasmid content as before (Labandeira-Rey and Skare, 2001) and those that mirrored strain ML23 were transformed with pBBE22*luc* and transformants were selected for resistance to kanamycin (Hyde *et al.*, 2011). Putative transformants were screened for the presence of pBBE22*luc* and total borrelial plasmids using methods described previously (Labandeira-Rey and Skare, 2001; Hyde *et al.*, 2011).

For genetic complementation, *bba33* and 210 bp 5' to the translation start site were cloned into the *Bam*HI and *Sal*I sites in the pBBE22*luc* shuttle vector to yield the final construct, pHZ300, using the oligonucleotides used are listed in **Table 3**. HZ001 was then made competent and electroporated with pHZ300 as described (Samuels, 1995). Transformants were selected for resistance to kanamycin and candidates were confirmed by PCR as indicated in **Fig. 3**. As before, borrelial plasmid DNA was accounted for using previously published methods (Labandeira-Rey and Skare, 2001). Only strains that contained the DNA profile of transformed ML23 were used for subsequent analyses.

Table 2. Strains and plasmid constructs used in this study.

Strain or plasmid	Genotype and/or characteristics	Source
E. coli strains		
Mach-1™-T1^{R}	ϕ80*lacZ*ΔM15 Δ*lacX*74 *hsdR* (r_K^-, m_k^+) Δ*recA1498 endA*1 *tonA*	Invitrogen
BL21 Star™ (DE3)	F$^-$ *ompT hsdSB* (rB$^-$mB$^-$) *gal dcm rne131* (DE3)	Invitrogen
BL21 Star™ (DE3) pLysS	F$^-$ *ompT hsdSB* (rB$^-$mB$^-$) *gal dcm rne131* (DE3) pLysS (Cam^R)	Invitrogen
Top10	F$^-$ *mcr*A Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139*, Δ(*ara leu*)7697 *galU gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
B. burgdorferi strains		
ML23	*B. burgdorferi* strain B31 clonal isolate missing lp25	(Labandeira-Rey and Skare, 2001)
ML23/pBBE22*luc*	Clonal isolate of strain B31 lacking lp25 containing *bbe22* and *B. burgdorferi* codon optimized *luc* gene under the control of a strong borrelial promoter (P_{flaB}-*luc*); parent strain	(Hyde *et al.*, 2011)
HZ001	ML23 Δ*bba33*::Str^R	This study
HZ001/pBBE22*luc*	ML23 Δ*bba33*::Str^R, containing *bbe22* and the *B. burgdorferi* codon optimized *luc* gene under the control of a strong borrelial promoter (P_{flaB}-*luc*)	This study
HZ001/pHZ300	ML23 Δ*bba33*::Str^R, containing *bbe22, bba33,* both under the control of their native promoters, and the *B. burgdorferi* codon optimized *luc* gene under the control of a strong borrelial promoter (P_{flaB}-*luc*)	This study

Table 2. Continued

Strain or plasmid	Genotype and/or characteristics	Source
Plasmids		
pCR2.1	pCR™2.1-TOPO® vector; Amp^R, Kan^R	Invitrogen
pKFSS1	*B. burgdorferi* shuttle vector containing P_{FlgB}-Str^R cassette; Spc^R in *E. coli*, Str^R in *B. burgdorferi*	(Frank et al., 2003)
pBBE22Gate	pBBE22 modified to be a Gateway destination vector containing *attL* and *attR* sites; Cam^R, Kan^R	(Weening et al., 2008) (Hyde *et al.*, 2011)
pBBE22*luc*	Borrelial shuttle vector containing *bbe22* and *B. burgdorferi* codon optimized *luc* gene under the control of a strong borrelial promoter (P_{flaB}-*luc*)	
pCR2.1Bactin	β-actin gene cloned into pCR2.1 vector; Kan^R	(Hyde *et al.*, 2011)
pCR2.1*recA*	1119 bp fragment, containing the *recA* gene, cloned into pCR2.1 vector; Kan^R	(Hyde *et al.*, 2011)
pET15b	Cloning vector for overexpression of genes to generate His-tagged recombinant protein; Amp^R	EMD Millipore
pSS008	*bba33* lacking its leader peptide (including the cysteine residue) was cloned into the *Nde*I and *Bam*HI sites of pET15b in order to produce His-tagged recombinant BBA33	This study
pHZ001	PCR amplicons containing sequences 1526 bp upstream of the start of *B. burgdorferi bba33*, the P_{flgB}-Str^R cassette from pKFSS1, and sequences 1527 bp downstream of the *bba33* stop codon, as well as pCR2.1, were specifically configured using Gibson assembly	This study
pHZ300	Intact *bba33* containing sequences 210 bp upstream and 129 bp downstream were cloned into the *Bam*HI and *Sal*I sites of pBBE22*luc*	This study

Infectivity studies and bioluminescent imaging

Infectivity studies were performed as previously described (Hyde et al., 2009). Briefly, 8-week-old C3H/HeN mice were inoculated with either 10^3 or 10^5 organisms of the *B. burgdorferi* parent strain ML23/pBBE22*luc*, the ∆*bba33* strain HZ001/pBBE22*luc*, or the genetic complement strain HZ001/pHZ300 by intradermal injection. For each dose and strain used, 4 mice were infected per group. Imaging of infected mice to detect bioluminescence in the aforementioned *B. burgdorferi* strains was done as described (Hyde *et al.*, 2011). After 21 days, the mice were sacrificed and inguinal lymph node, skin, heart, spleen, bladder and tibiotarsal joint tissues from each mouse were aseptically collected for *in vitro* cultivation and qPCR analysis of *B. burgdorferi* burden as described (Weening *et al.*, 2008; Hyde *et al.*, 2011). All animal experiments were performed in accordance to the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines. The Texas A&M University Institutional Animal Care and Use Committee (IACUC) approved all animal procedures used in this study.

DNA extraction of B. burgdorferi from infected tissues and qPCR analysis

Total DNA was isolated from mice skin, lymph node, heart and tibiotarsal joint samples using Roche High Pure PCR template preparation kit as previously described (Weening et al., 2008). Approximately 100 ng of total DNA was used

Table 3. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5' to 3')	Description
BamHI-A33us-recF	CCAAGCTTGGTACCGAGCTCGGATCCACAC GCGCTAGCATCCTTTAAATT	Primer pair used to amplify 1526 bp region upstream of *bba33* with a 22 bp 5' flanking region homologous to pCR2.1 upstream of the *Bam*HI site and a 3' flanking region homologous to the 5' region of the *flgB* promoter (P_{flgB})
flgB-A33us-recR	TAGGAAATCTTCCACGCCAATTGTTAAAGCT TTCTGTTTCTACGATATCG	
aadA-A33ds-recF	GTGAAAAAGTTTAAAAATCAGTTATTCTTCT ATAGGTTTTATTTCTG	Primer pair used to amplify the 1527 bp region downstream from *bba33* with a 21 bp 5' flanking region homologous to the *aadA* 3' end and a 32 bp 3' flanking region homologous to sequences downstream of the *Xho*I site of pCR2.1
A33ds-XhoI-recR	CGAATTGGGCCCTCTAGATGCATGCTCGAG CGGGTGTTGGAATTGGAAACGAACC	
flgB-F	CAATTGGCGTGGAAGATTTCC	Primer pair used to amplify P_{flgB}-Str^R cassette from pKFSS1
aadA-R	CTGATTTTTAAACTTTTTCACAAATAGG	
P1	GCCAGAGAGCGATATCGTAGAAAC	Primers P1 through P5 were used to confirm the *bba33* deletion in *B. burgdorferi* as well as complementation construct (see Fig. 2 and 3).
P2	AATAATAGGTTCATGAAAAATTTGTATG	
P3	CACAAATAGGGTCAAAATTTTGACC	
P4	TGCCCTATCAGAAATAAAACCTATAGAAG	
P5	GAAGTGCCTGGCAGTAAGTTG	

Table 3. Continued

Oligonucleotide	Sequence (5' to 3')	Description
A33-NdeI-5'F	ACGCCATATGTATTTAAATGATTTTTCTGGTATG	Primer pair used to amplify *bba33* lacking sequences that encode its leader peptide through the stop codon with *Nde*I (underlined) and *Bam*HI (bold) sites engineered for cloning into pET15b expression vector
A33-BamHI-3'R	ACGC**GGATCC**TTATTTTTGAATTAGTGCTAAAGCAC	
A33up210-BamHI-F	ACGC**GGATCC**GCCAGAGAGCGATATCGTAGAAAC	Primer pair used to amplify a region from 210 bp upstream and 129 downstream of *bba33* with BamHI (bold) and SalI (underlined) sites; cloned into pBBE22*luc*
A33dn129-SalI-R	ACGCGTCGACAAATGGCTAGGGTGGAATACAAAC	
β-Actin-F	ACGCAGAGGGAAATCGTGCGTGAC	Primer pair used for enumerating copies of mouse β -actin via qPCR (Pal et al., 2008)
β-Actin-R	ACGCGGGAGGAAGAGGATGCGGCAGTG	
nTM17FrecA	GTGGATCTATTGTATTAGATGAGGCT	Primer pair used for enumerating copies of *B.burgdorferi recA* via qPCR (Liveris et al., 2002; Weening et al., 2008)
nTM17RrecA	GCCAAAGTTCTGCAACATTAACACCT	

for each qPCR reaction. Quantitative real-time PCR analysis was conducted using the Applied Biosystems ABI 7900 HT system. *B. burgdorferi* genome copies and mammalian cell equivalents were determined using either the oligonucleotides nTM17FRecA and nTM17RRecA (Liveris et al., 2002; Weening et al., 2008) and primer set βactin-F and βactin-R (Pal et al., 2008), respectively. The bacterial burden was depicted as the number of *B. burgdorferi recA* per 10^6 β-actin copies.

Purification of recombinant BBA33

To overproduce and purify rBBA33, a construct lacking the first 51 bp of the *bba33* open reading frame (corresponding to the leader peptide) was amplified from *B. burgdorferi* genomic DNA using primer set A33-NdeI-5'F and A33-BamHI-3'R (**Table 3**) and cloned in-frame into pET15b following its digestion with *Nde*I and *Bam*HI. The resulting construct, designated pSS008, encodes an amino terminal His-tagged version of BBA33. To affinity purify His-tagged BBA33, pSS008 was transformed into BL21 Star™(DE3) pLysS, grown to early log phase, and the His-tagged BBA33 produced following induction with 0.2 mM IPTG for 16 hrs at 15°C. The cells were harvested by centrifugation, the pellet frozen, and lysed via French pressure cell treatment. The soluble fraction, containing the His-tagged BBA33 protein, was applied to HisPur Cobalt resin (Thermo scientific) and affinity purified as outlined by the manufacturer.

Rabbit polyclonal antiserum to BBA33 was obtained following immunization of New Zealand white rabbits via an IACUC approved regimen at the Comparative Medicine Program, Texas A&M University.

SDS-PAGE and immunoblotting

B. burgdorferi protein lysates were resolved by SDS–PAGE (Laemmli, 1970) and gels were either stained with Coomassie Brilliant Blue R-250 (Sigma Aldrich, St Louis, MO, USA) or transferred to PVDF membranes and immunoblotted as described (Weening et al., 2008). Primary antibodies were used at the following dilutions: anti-BBA33 at 1:2000; anti-His_6 (GE Healthcare) at 1:6000; anti-OspC at 1:4 x 10^6 (a kind gift from R. Gilmore); anti-PncA at 1:1500 (generously provided by J. Seshu); anti-P66 at 1:1000 (kindly provided by Sven Bergström); or anti-FlaB at 1:20,000 (Affinity Bioreagent, Golden, CO). Prior to its use for Western immunoblotting, the BBA33 antiserum was adsorbed against HZ001 borrelial lysates to reduce the amount of non-specific antibodies, as reported previously (Skare et al., 1999). Appropriate secondary antibodies, with horseradish peroxidase (HRP) conjugates [anti-mouse HRP (Invitrogen, Carlsbad, CA) or anti-rabbit HRP (Amersham, Piscataway, NJ), both diluted 1:4000] were used to detect immune complexes, the membranes washed extensively in PBS, 0.2% Tween-20, and developed using the Western Lightning Chemiluminescent Reagent plus system (Perkin Elmer, Waltham, MA, USA).

Proteinase K accessibility assay

B. burgdorferi strain ML23 pBBE22*luc* was grown under inducing conditions (37˚C, 5% CO_2, pH 6.8) and harvested by centrifugation at 5,800 x *g*, and washed twice with PBS. The cell pellet was re-suspended in 0.5 ml of either PBS alone, PBS with proteinase K (to a final concentration of 200 µg ml^{-1}), or PBS/proteinase K with 0.05% Triton X-100. All samples were incubated at 20°C for 40 min. Reactions were terminated by the addition of phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 1 mM. Cells were again pelleted by centrifugation (9,000 x *g* for 10 min at 4°C), washed twice with PBS containing 1 mM PMSF, and re-suspended in Laemmli sample buffer (Laemmli, 1970). Samples were run on SDS-PAGE gel and probed with anti-BBA33, anti-P66, and anti-FlaB antibodies, respectively.

Triton X-114 phase partitioning

B. burgdorferi strain ML23 pBBE22*luc* was grown under inducing conditions (37˚C, 5% CO_2, pH 6.8) and subjected to Triton X-114 phase partitioning as previously described (Skare et al., 1995).

Solid-phase phage display screening

A phage library displaying random cyclic 8-mer peptides (CX_8C; X is any amino acid) was used for the screening against rBBA33 immobilized onto microtiter wells overnight at 4˚C (at 1 µg/well in PBS). After the wells were washed

twice with PBS and blocked with PBS containing 3% BSA for 1 hr at 23˚C, 10^9 PFU in 50 µl PBS containing 2% BSA were added to the wells (Barbu et al., 2010). After 2 hours at 23˚C, the wells were washed 6 times with PBS containing 0.1% Tween 20 and 3 times with PBS, and phage were recovered by bacterial infection as described (Smith and Scott, 1993). Phage recovered after the second and third round of selection were subjected to dideoxy sequencing and the resulting deduced amino acid sequences compared in the existing NCBI protein database.

In vitro ECM binding assay

Maxinunc microtiter plates (eBiosciences) were coated with 0.5 µg human collagen I (Corning), human collagen VI (Abcam), bovine fibronectin (Corning), mouse collagen IV (Corning), and mouse laminin (Corning) at 4°C for overnight, and blocked with 3% BSA for 1 hour at 37 °C. Recombinant His-tagged BBA33 protein was serially diluted 1:2 starting from 4 µM down to 62.5 nM in PBS, 0.1% Tween-20, and added to coated wells in triplicate and incubated for 1 hour at 37 °C. After 5 washes in PBS, 0.1% Tween-20, a 1:6000 dilution of monoclonal antibody directed against poly-His (His_6) was added to each well and incubated for 1 hr at 37 °C. Following 5 washes in PBS, 0.1% Tween-20, a 1:4000 dilution of horseradish peroxidase (HRP) conjugated anti-mouse IgG was added to each well and incubated for 1 hr at 37 °C. The wells were then washed in PBS, 0.1% Tween-20, after which 3,3',5,5'–tetramethylbenzidine (TMB) was added as

substrate. The enzymatic reaction was stopped after 3 min using 0.16 M sulfuric acid (Thermo scientific) and the absorbance at 450 nm was determined.

***B. burgdorferi* whole cell adherence assay**

Poly-D-lysine pre-coated coverslips (Corning Biocoat) were coated with 2 μg human collagen VI or mouse laminin and incubated at 4°C overnight. The coverslips were washed thoroughly in PBS to remove excess unbound proteins. The coverslips were then blocked with 3% BSA at 37 °C for 1 hr. The *B. burgdorferi* parent strain ML23 pBBE22*luc*, the ∆*bba33* strain HZ001/pBBE22*luc*, and the genetic complement strain HZ001/pHZ300 were all grown to exponential phase at either 32°C, 1% CO_2, pH 7.6 (non-inducing conditions) or 37°C, 5% CO_2, pH 6.8 (inducing conditions). The resulting cells were then harvested by centrifugation at 5,800 × *g* and washed with BSK-II medium without serum three times, and diluted to 10^7 organisms/ml in BSK-II medium without serum. The resulting *B. burgdorferi* cells, in 0.1 ml volumes, were applied onto the coverslips and incubated for 2 hr at 32°C. Unbound bacteria were removed from the coverslips by gentle washing with PBS; this wash step was repeated 10 times. The coverslips were applied to a glass slide and attached bacteria were counted by dark field microscopy. Binding was scored as the average number of bacteria bound per field based on 10 random fields per slide.

Statistical analysis

For real-time qPCR analysis, a one-tailed Mann–Whitney's *t*-test was performed between the strains indicated. For ELISA and bioluminescent assays, Two-way analyses of variance (ANOVA) were performed respectively among variables. Tukey's post-test was used to determine *P*-values between coated proteins. Statistical significance was accepted when the *P* values were less than 0.05 for all statistical analyses employed.

CHAPTER III

Borrelia burgdorferi BBK32 INHIBITS THE CLASSICAL PATHWAY BY BLOCKING ACTIVATION OF THE C1 COMPLEX COMPLEX∗

Introduction

In the prior Chapter, we determined that BBA33 bound to collagen. Proteins that bind to collagens often also recognize the classical complement protein C1q, which is a component of the C1 complex. Microbial proteins from pathogens that bind C1q can also interfere with complement dependent killing mechanisms by inhibiting classical complement activation. As such, given the profound phenotype in borrelial cells lacking BBA33, we were interested in determining if BBA33 could bind C1q and block C1 activation. We determined (in collaboration with Magnus Höök's group) that BBA33 did bind to C1q but did not inhibit the classical complement pathway. However, we did find that the BBK32 protein, the known fibronectin adhesin (Fischer et al., 2006; Kim et al., 2004; Norman et al., 2008; Probert and Johnson, 1998; Seshu et al., 2006), which was added initially as a control, was able to potently inhibit the classical pathway at a

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low K_d value. The characterization of the BBK32::C1 interaction serves as the basis for this Chapter of the dissertation.

Blood-borne pathogens, or those that traffic in interstitial fluid or lymphatics, which lack endogenous regulators of complement activity, must adopt strategies to successfully evade complement attack. One such pathogen, *Borrelia burgdorferi*, is the etiologic agent of Lyme disease and is the leading cause of vector-borne illness in the United States according to the Centers for Disease Control and Prevention (CDC). Lyme disease is often accompanied by a local erythema migrans lesion and can lead to severe clinical outcomes such as carditis, neurological dysfunction, and arthritis (Radolf et al., 2012; Stanek et al., 2012). *B. burgdorferi* is transmitted to humans via the bite of infected hard ticks. During the tick's blood meal, spirochetes enter the mammalian host and subsequently disseminate to remote tissues (Radolf et al., 2012; Stanek et al., 2012). If therapeutic intervention is not sought, *B. burgdorferi* is able to persistently colonize a large number of tissues including joint, skin, heart, and the central nervous system (Radolf et al., 2012; Stanek et al., 2012). *B. burgdorferi* appears to avoid complement-mediated killing from the alternative pathway by expressing a group of virulence factors known as Csp proteins (CspA and CspZ) and those from the OspE/F family (Alitalo et al., 2002; Hellwage et al., 2001; Kenedy and Akins, 2011; Kenedy et al., 2009; McDowell et al., 2003a; Rogers and Marconi, 2007; Rogers et al., 2009). These proteins are also referred to as complement regulator-acquiring surface proteins (CRASPs) (Bykowski et al., 2008; de Taeye

et al., 2013). These bacterial surface proteins recruit human factor H, factor H-like protein 1, and factor H-related proteins, which serve as the major endogenous negative regulators of the alternative pathway (Brooks et al., 2005; Hallström et al., 2013; Kenedy et al., 2009; Kraiczy et al., 2000; McDowell et al., 2003a; de Taeye et al., 2013). In addition, human factor H is also recruited to the surface of relapsing fever *Borrelia* spp. where similar alternative pathway inhibition would occur (Hovis et al., 2004; McDowell et al., 2003b). By hijacking these key host complement regulatory molecules, *B. burgdorferi,* as well as other *B. burgdorferi sensu lato* isolates, subverts the deleterious effects of alternative pathway activation.

Activation of the classical pathway has previously been shown for Lyme disease spirochetes (Dam et al., 1997; Kochi and Johnson, 1988) and studies employing mouse models deficient in factor H, factor B, or C3 have shown that the classical pathway and/or lectin pathway play significant roles in controlling early stages of borrelial infection (Woodman et al., 2007). Indeed, the importance of spirochetal strategies to subvert classical pathway activation is underscored by the ability of *B. burgdorferi* as well as the relapsing fever spirochetes *B. recurrentis* and *B. duttonii* to recruit the host classical pathway regulators C4b-binding protein and/or C1 esterase inhibitor (C1-INH) to their surface via interactions with specific borrelial lipoproteins (Grosskinsky et al., 2010; Meri et al., 2006; Pietikäinen et al., 2010). Our studies with BBA33 binding to collagen (Chapter II) led us to look at the ability of this protein to recognize C1q, the classical complement protein with

collagen-like motifs, and ask whether BBA33 could inhibit the activation of the C1q-containing C1 complex. Although BBA33 does bind to C1q, it did not affect C1 activation. However, in this analysis, we found that BBK32 functions as a potent and specific inhibitor of the classical pathway capable of forming a high-affinity interaction with C1. We then localized the anti-complement activity of BBK32 to the C-terminal region and demonstrated a molecular mechanism by which BBK32 non-covalently inactivates the central classical pathway initiating serine protease C1r. To our knowledge, BBK32 represents the first example of a C1r specific inhibitor of biomolecular origin and is the first noncovalent protein inhibitor of the C1 complex to be described. Thus, this work significantly expands our knowledge of how pathogens recognize and evade human innate immunity by defining a new mechanism by which the pathogen *B. burgdorferi* prevents activation of the classical pathway of complement.

Results

The Borrelia burgdorferi lipoprotein BBK32 interacts with the first component of human complement, C1

In light of the apparent ability of *B. burgdorferi* to suppress the classical pathway (discussed above), we hypothesized that novel interactions exist between *B. burgdorferi* surface proteins and the classical pathway initiating enzyme complex, complement C1. To explore this hypothesis we adopted a Far Western immunoblot approach designed to probe for interaction of *B. burgdorferi*

B31 lysate proteins with C1. The initial profile showed that biotinylated C1 specifically recognizes borrelial proteins with apparent molecular masses of 17, 28, and 48 kDa (**Fig. 12A**). We also tested the reactivity of lysates harvested from cultures grown under conditions that require the Rrp2-RpoN-RpoS regulatory system for their induction (Boardman et al., 2008; Caimano et al., 2007; Hubner et al., 2001; Yang et al., 2003) (e.g., pH 6.8 relative to conventional growth conditions of pH 7.6) and thus mimic, in part, some aspects of the mammalian host environment (Boardman et al., 2008; Burtnick et al., 2007; Caimano et al., 2007; Hubner et al., 2001; Yang et al., 2000, 2003). Given that the 48-kDa band was near the known SDS-PAGE migration position of the borrelial lipoprotein BBK32 (Probert and Johnson, 1998), we also tested lysates originating from cells that lacked an intact *bbk32* locus (*bbk32*::Str^R; (Seshu et al., 2006)) (**Fig. 12A**). These experiments show that the 48-kDa band is inducible under mammalian host-like conditions and is also completely absent from *bbk32*::Str^R lysates. The two lower molecular weight bands did not change in intensity in response to altered growth conditions and remained present in the *B. burgdorferi* lacking intact *bbk32*, indicating that they were not proteolytic fragments of BBK32 and instead are distinct protein species (**Fig. 12A**). Immunoblot analysis also confirmed that the 48-kDa C1-reactive species co-migrated with BBK32 in the parent strain and was missing in the *bbk32*::Str^R mutant (**Fig. 12B**).

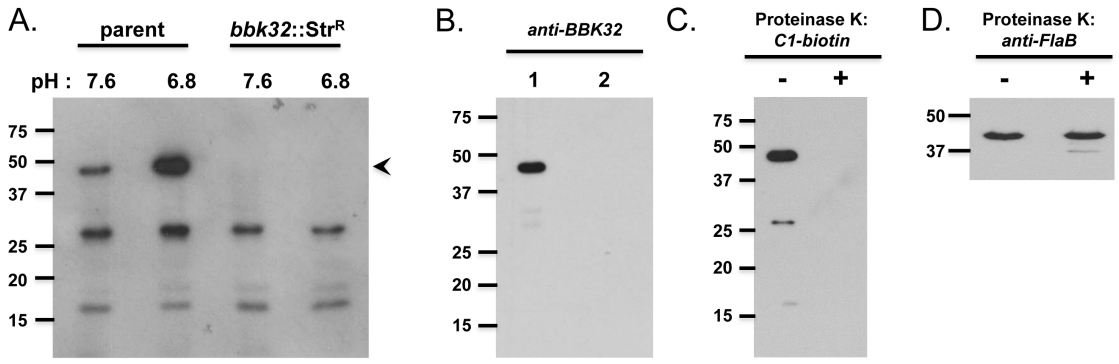

Figure 12. *B. burgdorferi* expresses surface-exposed proteins that bind complement C1. Far Western Blot analysis of *B. burgdorferi* cell lysates probed with complement C1. (A) Biotinylated C1 was used to probe cell lysates from *B. burgdorferi* B31 strain ML23 grown under conventional (pH 7.6) or induced (pH 6.8) conditions. Proteins of apparent molecular weights 17, 28, and 48 kDa were capable of binding C1, while the 48-kDa band alone was inducible. Lysates from a strain lacking an intact *bbk32* locus (*bbk32*::Str^R) lacked the 48-kDa band. (B) Immunoblot analysis with anti-BBK32 demonstrated that the 48-kDa protein co-migrates with the C1 reactive species in the parent strain (lane 1) and is missing from the *bbk32*::Str^R mutant (lane 2) providing further support that it is BBK32. Both strains shown here were grown at 37˚C, pH 6.8, e.g., under inducing conditions. (C) To determine if the C1 binding proteins present in borrelial lysates were surface exposed, a proteinase K assay was employed. All three bands are absent from proteinase K treated samples. (D) The subsurface endoflagellar protein FlaB are identical between mock and protease treated cells, indicating that the *B. burgdorferi* cells retained structural integrity.

Next, we were interested in determining if the C1-binding proteins were surface exposed in *B. burgdorferi*. To this end, we used a proteinase K accessibility assay that serves as a readout for the surface localization of borrelial proteins (Barbour et al., 1984; Probert et al., 1995). The results indicated that all three C1 reactive proteins were eliminated following protease treatment suggesting that they are all on the outer surface of *B. burgdorferi* (**Fig. 12C**). To address the integrity of the *B. burgdorferi* cells, we tested whether the subsurface endoflagellar structural protein FlaB was affected by the addition of proteinase K. The levels of FlaB between mock and protease treated cells were not different (**Fig. 12D**), indicating that the *B. burgdorferi* cells used in this experiment were structurally intact.

The C-terminal globular domain of BBK32 binds C1 with high-affinity in a calcium-dependent manner

The identity of the 17 and 28-kDa proteins targeted by biotinylated C1 in the Far Western assay remain unclear at this time. However, in addition to gel migratory position, three lines of evidence suggested that the identity of the 48-kDa band was indeed BBK32; (i) it is induced under mammalian-like conditions (**Fig. 12A**), (ii) it is surface exposed (**Fig. 12C**), and (iii) it is not detected in samples containing the *bbk32*::Str^R allele (**Fig. 12A** and **12B**). To further investigate a potential C1::BBK32 interaction we next produced a recombinant form of BBK32 (residues 21 to 354) which lacks only the 20 residue signal peptide

(Probert and Johnson, 1998), and immobilized this “full-length” BBK32 (referred to as BBK32-FL hereafter) protein on the surface of a Biacore sensor chip. Surface plasmon resonance (SPR) was used to quantitatively measure the interaction of purified C1 with immobilized BBK32-FL in a running buffer of HBS-T-Ca^{2+}. Strong and dose-dependent binding was observed (**Fig. 13A**) and kinetic evaluation of the resulting sensorgrams indicates that BBK32-FL binds C1 with high affinity, as a dissociation constant (K_D) of 3.9 nM (**Table 4**) was calculated.

BBK32 has long been known for its ability to bind the extracellular matrix glycoprotein fibronectin (Fn) (Fischer et al., 2006; Probert and Johnson, 1998; Probert et al., 2001). The Fn/BBK32 interaction is primarily mediated by anti-parallel β-strand addition of residues 126-190 originating from the intrinsically disordered N-terminal region of BBK32 with Fn type I domains along an extended region of Fn (Probert et al., 2001; Raibaud et al., 2005). In addition to Fn, glycosaminoglycans (GAGs) also act as a mammalian host ligand for BBK32 and are recognized by a distinct N-terminal binding site (BBK32 residues 45-68) (Lin et al., 2014). In contrast, the C-terminal region of BBK32 (residues 206-354 and hereafter referred to as BBK32-C) is a highly basic globular domain rich in α-helical secondary structure (Kim et al., 2004), which to date has not been ascribed a specific function. To determine if the binding site for C1 on BBK32 could be localized to the N-terminal region of BBK32 (residues 21-205, and hereafter referred to as BBK32-N) or the globular C-terminal region, we next immobilized recombinant versions of BBK32-N and BBK32-C on the surface of an SPR sensor

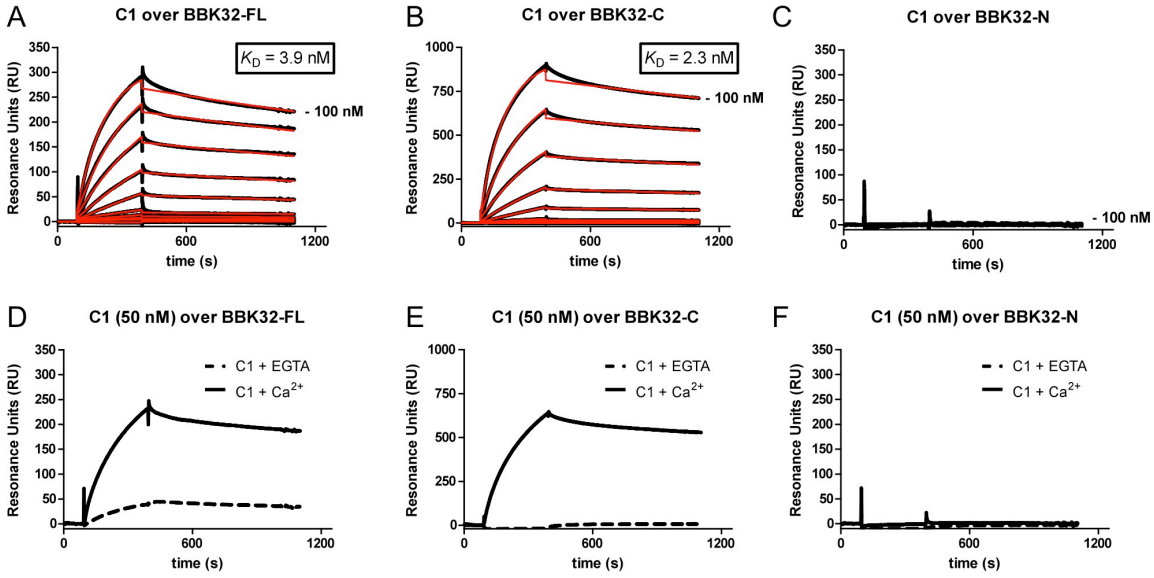

Figure 13. The C-terminal domain of BBK32 mediates a high-affinity and calcium-dependent interaction with complement C1. SPR was used to measure the direct binding of C1 to BBK32 proteins. A twofold dilution series of C1 (0.2 – 100 nM) was injected over immobilized (A) BBK32-FL, (B) BBK32-C, or (C) BBK32-N in a running buffer of HBS-T-Ca^{2+} and the SPR response was recorded (black lines). BBK32-FL and BBK32-C bind C1 with high affinity while BBK32-N exhibits no detectable response. Kinetic analysis of the resulting sensorgrams was performed and fits are represented as red traces. The derived binding constants and fitting statistics are reported in Table 1. Sensorgrams from a representative injection series are shown and all experiments were conducted in triplicate. BBK32/C1 interaction is strongly calcium dependent as evidenced by greatly diminished binding in the presence of the calcium chelator EGTA (D-F).

Table 4. SPR: BBK32/C1 and C1r binding parameters.

Immobilized Ligand	Analyte	K_D (nM)	k_a (M^{-1} s^{-1})	k_d (s^{-1})
BBK32-FL	C1	3.9 ± 0.31	(7.2 ± 0.24) x 10^{4}	(2.8 ± 0.14) x 10^{-4}
	C1r enzyme	15 ± 7.5	(1.9 ± 1.1) x 10^{4}	(2.2 ± 0.85) x 10^{-4}
BBK32-C	C1	2.3 ± 0.11	(8.4 ± 0.21) x 10^{4}	(1.9 ± 0.01) x 10^{-4}
	C1r enzyme	32 ± 11	(3.9 ± 2.2) x 10^{3}	(1.1 ± 0.46) x 10^{-4}

The dissociation constant (K_D), association rate constant (k_a) and dissociation rate constant (k_d) were calculated by performing kinetic analysis for each interaction. All experiments were performed between three and five times and errors are reported as the mean ± SEM.

chip. Intriguingly, no detectable response was measured when C1 was injected over the BBK32-N fragment (**Fig. 13C**); however, the C-terminal domain of BBK32 retained a high-affinity for C1 (K_D = 2.3 nM) (**Fig. 13B, Table 4**). Furthermore, BBK32-C has a very similar kinetic profile (k_a = 8.4 x 10^4 $M^{-1}s^{-1}$, k_d = 1.9 x 10^{-4} s^{-1}) to that measured for C1::BBK32-FL (k_a = 7.2 x 10^4 $M^{-1}s^{-1}$, k_d = 2.8 x 10^{-4} s^{-1}). The interaction of both BBK32-FL and BBK32-C with C1 was strongly dependent on calcium as binding was nearly abolished when C1 was injected in a buffer containing the calcium chelator ethylene glycol tetraacetic acid (EGTA) (**Fig. 13D-F**). Taken together, these results demonstrate that the C-terminal region of BBK32 forms a tight calcium-dependent interaction with human complement C1.

The C-terminal domain of BBK32 specifically and potently blocks the classical pathway of human complement

Given the apparent high-affinity interaction between BBK32 and C1 we sought to determine if BBK32 modulates complement activation. To this end, we evaluated the effect of various concentrations of BBK32-FL, BBK32-N and BBK32-C in an ELISA-based assay of complement function. When conditions specific for the classical pathway activation were used, both BBK32-FL ($IC_{50,\ C3b}$ = 34 nM, $IC_{50,\ C4b}$ = 34 nM) and BBK32-C ($IC_{50,\ C3b}$ = 4.7 nM, $IC_{50,\ C4b}$ = 5.6 nM) potently inhibited the generation of the downstream complement activation products C3b and C4b (**Fig. 14 A-B, Table 5**). In contrast, BBK32-N failed to inhibit the classical pathway activation at any concentration used. Importantly,

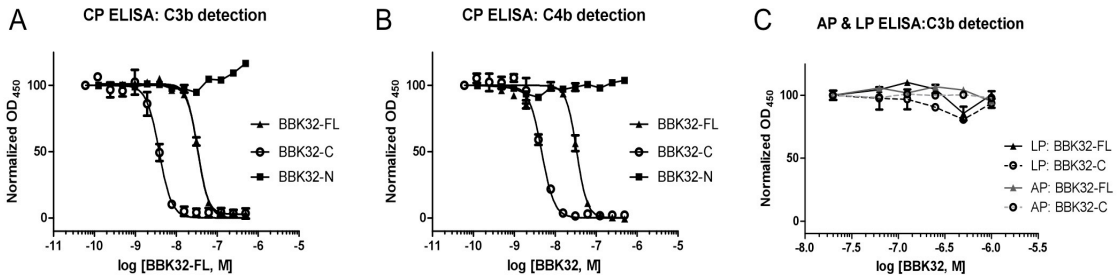

Figure 14. The C-terminal domain of BBK32 specifically inhibits the classical pathway in a dose-dependent manner in an ELISA based assay of complement function. classical pathway was selectively activated by immobilization of human IgM followed by incubation of 1% human serum in GHB^{++} buffer in the presence of varying concentrations of BBK32 proteins. Monoclonal antibodies were used to detect the deposition of the complement activation products (A) C3b or (B) C4b. While BBK32-FL and the BBK32-C potently inhibit the classical pathway in a dose-dependent manner, BBK32-N showed no apparent inhibitory activity. In contrast, when conditions were used to select for (C) alternative pathway activation (LPS coating, 20% serum, GHB°, Mg-EGTA) or lectin pathway activation (mannan coating, 1% serum, GHB^{++}) no significant inhibition was detected for up to 1 μM concentrations of any BBK32 protein derivative. Wells containing serum only or where serum was replaced with buffer were treated as 100% and 0% signal, respectively. All experiments were performed a minimum of three times, errors are reported as the mean ± SEM, and calculated IC_{50} values are reported in Table 5.

Table 5. IC_{50} values for inhibition of the classical pathway by BBK32 proteins.

Inhibitor	Assay	IC_{50} (nM)	R^2
BBK32-FL	ELISA: C3b detection	34 ± 2.2	0.997, 0.997, 0.996
	ELISA: C4b detection	34 ± 3.6	0.948, 0.987, 0.969
	Hemolytic	110 ± 11	0.995, 0.995
	C1r enzyme activity	670 ± 280	0.982, 0.976
BBK32-C	ELISA: C3b detection	4.7 ± 2.2	0.970, 0.961, 0.971
	ELISA: C4b detection	5.6 ± 2.0	0.927, 0.989, 0.983
	Hemolytic	60 ± 4.9	0.977, 0.987, 0.967, 0.946
	C1r enzyme activity	540 ± 91	0.983, 0.956, 0.965, 0.998
BBK32-N	ELISA: C3b detection	No inhibition	NA
	ELISA: C4b detection	No inhibition	NA
	Hemolytic	No inhibition	NA
	C1r enzyme activity	No inhibition	NA

IC_{50} values were obtained by nonlinear regression analysis using a four-parameter variable slope fit and constraining the bottom and top values to 0 and 100 respectively. The 'goodness of fit' parameter R^2 is provided for each individual fit of replicate experiments. NA = not applicable.

when conditions were used to selectively activate the alternative or lectin pathway, no significant effect could be measured at concentrations up to 1 μM of BBK32-FL or BBK32-C (**Fig. 14C**).

To further investigate their complement inhibitory activities, we next measured residual complement-mediated hemolysis in the presence of 1 μM BBK32 proteins. Strikingly, 1 μM BBK32-FL or BBK32-C conferred nearly 100% protection to sensitized sheep red blood cells in the presence of complement (i.e., normal human serum) using a standard assay of the classical pathway-mediated hemolysis (CP50) (**Fig. 15A**). Furthermore, BBK32 inhibited the classical pathway in a dose-dependent manner with calculated IC_{50} values of 110 nM and 60 nM for BBK32-FL and BBK32-C, respectively (**Fig. 15B and Table 5**). Similar inhibition of the classical pathway could not be detected for the N-terminal BBK32 fragment (**Fig. 15A-B**). Consistent with the ELISA based complement assay (**Fig. 14C**), BBK32 proteins did not have an effect on alternative pathway-mediated hemolysis of rabbit blood cells (**Fig. 15C**). Taken together, these results demonstrate that BBK32 acts as a potent and specific inhibitor of the classical pathway of human complement and this inhibitory activity locates to the C-terminal globular region of BBK32.

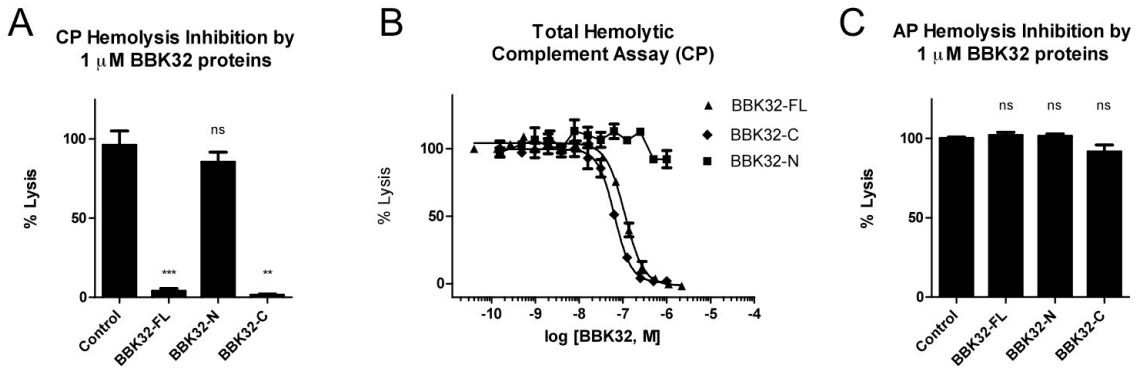

Figure 15. The C-terminal domain of BBK32 specifically inhibits the classical pathway-mediated hemolysis. (A) The effect of 1 μM BBK32 proteins on the classical pathway - mediated hemolysis was assessed using a standard assay of complement hemolytic function (CP50). (B) While BBK32-N exhibited no measurable effect, BBK32-FL and BBK32-C abrogated nearly all hemolytic activity in a dose-dependent manner. (C) In contrast, BBK32 proteins fail to inhibit alternative pathway-mediated hemolysis. Measures of statistical significance in (A) and (C) were determined by use of an unpaired *t* test of each experimental series versus buffer control. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$; ns, not significant. All experiments were performed between two and four times and errors are reported as the mean ± the standard error of the mean (SEM). Calculated IC_{50} values for the dose-dependent inhibition of the classical pathway hemolysis are reported in Table 5.

BBK32 interacts directly with the C1 subunit, C1r protease, in a calcium-dependent manner

The data presented above indicate that BBK32 can form a high-affinity interaction with C1 and that this interaction results in a specific inhibition of the classical pathway of human complement. The calcium-dependence of the C1::BBK32 interaction (**Fig. 13 D-F**) raised the distinct possibility that BBK32 was capable of recognizing only the fully formed calcium-mediated C1 complex rather than binding to an individual C1 subunit with high-affinity. To determine if this was the case we used SPR and monitored the response generated by individual 50 nM injections of C1q, C1r enzyme, C1s enzyme, and C1s proenzyme over the surface of a BBK32-FL or BBK32-C SPR biosensor (**Fig. 16 A and B**). Surprisingly, we found that BBK32 binds specifically to C1r, but not to other C1 components (**Fig. 16 A and B**). In agreement with C1/BBK32 binding activity, BBK32-C binds C1r with similar affinity to that of full-length BBK32 ($K_{D,\ C1r/BBK32\text{-}FL}$ = 15 nM vs. $K_{D,\ C1r/BBK32\text{-}C}$ = 32 nM) (**Fig. 16 C, D and Table 4**). The ability of BBK32 to interact with C1r could also be observed using a Far Western approach where *B. burgdorferi* lysates were probed with biotinylated C1r (**Fig. 16E**). A band corresponding to the 47-kDa BBK32 protein was detected in the parent lysate but was absent in the lysate from the *bbk32* mutant strain. Intriguingly, two additional "non-BBK32" bands were also capable of interacting with C1r using this technique. While the identities of these proteins are currently unknown, we note the presence of a

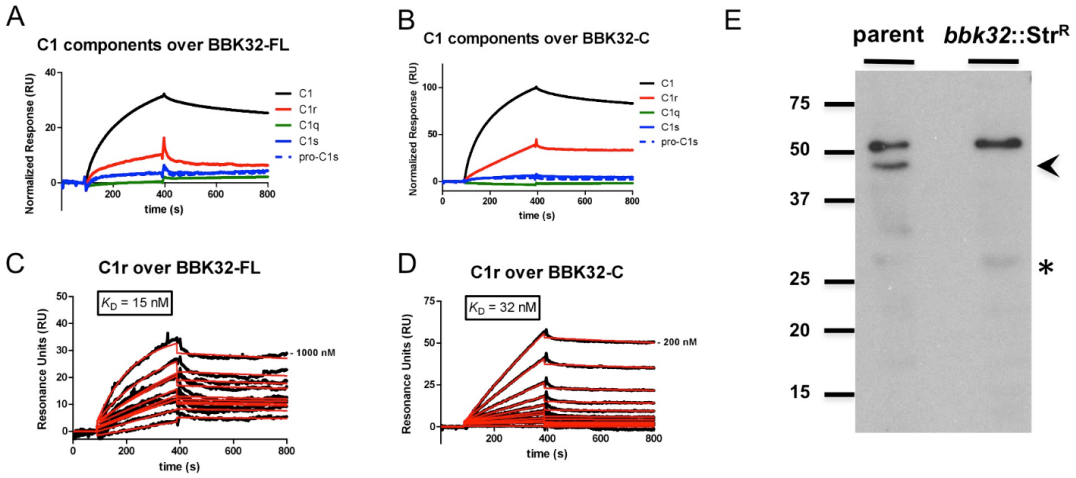

Figure 16. BBK32 forms a calcium-dependent, high-affinity interaction with the C1 subunit, complement C1r. SPR analysis was used to evaluate C1 component binding to BBK32-FL or BBK32-C. C1 or individual protein constituents of the C1 complex were injected at a fixed 50 nM concentration over the surface of immobilized (A) full-length BBK32-FL or (B) BBK32-C. As SPR signal is directly proportional to molecular weight, the resulting sensorgrams were normalized using the molecular weight of each analyte species. Only injection of C1 or C1r enzyme resulted in significant binding. The SPR response (black lines) was recorded for a twofold dilution injection series of C1r enzyme in a running buffer of HBS-T-Ca^{2+} over immobilized (C) BBK32-FL or (D) BBK32-C. Five independent injection series were collected and sensorgrams shown are from a representative experiment. Red traces represent fits from kinetic evaluation of the injection series. The derived binding constants and fitting statistics are reported in Table 1. (E) Far Western Blot analysis was used to assess interaction of *B. burgdorferi* lysates with C1r enzyme. Biotinylated C1r enzyme was used to probe cell lysates from *B. burgdorferi* B31 strain ML23 grown under conventional (pH 7.6) conditions. Proteins of apparent molecular weights 28, and 48, and 54 kDa were capable of binding C1r in the parent strain, while the 48-kDa band alone was absent from the strain lacking an intact *bbk32* locus (*bbk32*::Str^R). An arrowhead denotes the band corresponding to BBK32 while an asterisk denotes a band migrating to a position observed in the C1 Far Western (Fig. 12A).

28-kDa-reactive band present in the parent strain (denoted with an asterisk) that matches the migration position of a C1 reactive band (**Fig. 12**).

Kinetic evaluation of the SPR data suggest that both the BBK32-FL and the BBK32-C proteins recognize C1 preferentially over C1r with an increased affinity being attributed primarily to an increase in the association rate (**Table 4**). Nonetheless, these binding data strongly suggest that the major BBK32 binding site on the C1 complex is mediated by C1r. Interestingly, we found that the C1r/BBK32 interaction was also dependent on calcium (**Fig. 17A, B**). C1r itself possesses multiple calcium binding sites and the structural and functional consequences of C1r/calcium binding are complex (Bally et al., 2009; Busby and Ingham, 1990; Major et al., 2010; Thielens et al., 1999). For example, calcium binding of C1r results in large-scale conformational changes of the C1r CUB2 domain from a disordered structure in the absence of calcium to a fully folded structure in its presence (Major et al., 2010). The dependence of the BBK32::C1r interaction on calcium suggests that BBK32 recognizes a calcium-dependent C1r conformation, which further implicates the calcium-dependent C1 complex as the physiologically relevant BBK32 ligand.

BBK32 inhibits C1r autoactivation and prevents the enzymatic cleavage of C1s proenzyme

The ability of BBK32 to bind both C1 and C1r and to specifically inhibit the classical pathway of complement suggested that BBK32 interfered with the

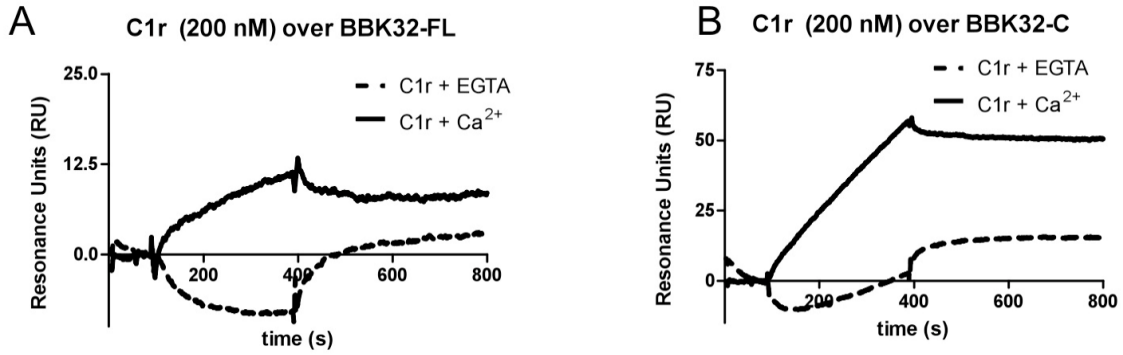

Figure 17. The interaction of BBK32 with C1r is calcium dependent. As was observed for the C1 complex, the interaction of C1r with (A) full-length BBK32-FL or (B) BBK32-C is strongly dependent on calcium as judged by SPR.

enzymatic activity of the C1r protease. To determine if this was the case we first measured the effect of various concentrations of BBK32-FL, BBK32-C, and BBK32-N on the *in vitro* conversion of C1s proenzyme by previously activated C1r enzyme (**Fig. 18A**). Indeed, both BBK32-FL and BBK32-C inhibited C1r in a dose-dependent fashion, while BBK32-N failed to inhibit up to a 10 μM final concentration. Quantification of these data by densitometry was performed by evaluating the peak intensity of the C1s proenzyme band (**Fig. 18B**) and calculated IC_{50} values are reported in **Table 5**. BBK32 proteins do not affect the activity of previously activated C1s (C1s enzyme) as C4 is cleaved by C1s in the presence of 1 μM BBK32 proteins (**Fig. 18C**). These results indicate that BBK32 specifically inhibits C1r enzyme by preventing the processing of its natural substrate, the C1s proenzyme.

When C1 is incubated at 37°C, it becomes activated by the autocatalysis of C1r proenzyme and subsequent C1r enzyme cleavage of the C1s proenzyme (Ziccardi, 1982). To determine if BBK32 affects C1r and C1s zymogens within the C1 complex, we incubated BBK32 proteins (5 μM) with C1 (40 nM) for two hours at 37°C and co-immunoprecipitated (co-IP) C1 using a C1q monoclonal antibody. Bound fractions were subjected to SDS-PAGE, followed by Western immunoblot analysis, and the presence of C1r, C1s, C1q, or BBK32 was then assessed.

When reactions were probed with C1r antibody (**Fig. 19A**), a processed form of C1r was observed for the buffer only control as indicated by the presence

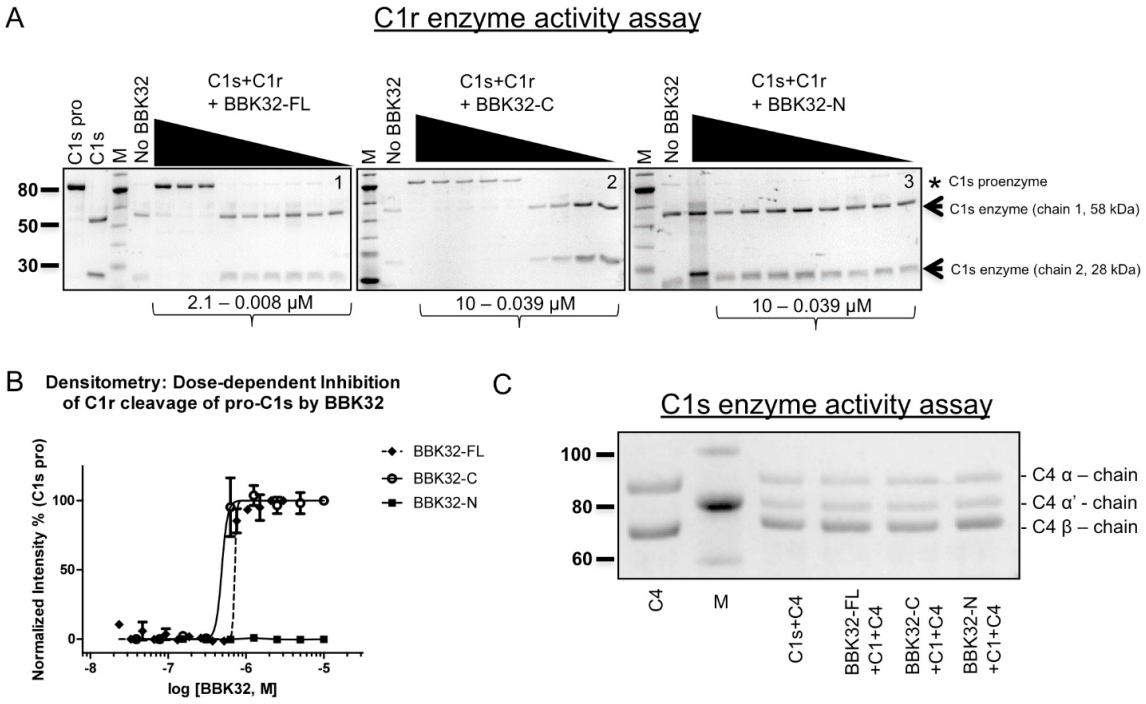

Figure 18. BBK32 inhibits the enzymatic cleavage of C1s proenzyme by C1r enzyme. (A) The ability of BBK32 to inhibit the *in vitro* proteolytic cleavage of C1s proenzyme by 50 nM of activated C1r enzyme was assessed by monitoring for the presence of C1s proenzyme (single 86-kDa chain; asterisk) or activated C1s enzyme (58-kDa chain 1 and 28-kDa chain 2; arrows) by SDS-PAGE. Overnight reactions were incubated at 37°C in HBS-Ca^{2+} in the presence of various concentrations of BBK32-FL (gel 1), BBK32-C (gel 2), or BBK32-N (gel 3). In the absence of BBK32, C1r enzyme converts 100% of C1s proenzyme to C1s enzyme under the conditions used (see "No BBK32" lanes on gels 1-3). BBK32-FL and BBK32-C inhibited C1r activity in a dose-dependent manner, whereas, BBK32-N failed to inhibit C1r up to a final concentration of 10 µM. All C1r activity assays were conducted a minimum of three times and representative gel images are shown. (B) Densitometry was performed and the normalized peak intensity of the band corresponding to C1s proenzyme was plotted against the concentration of BBK32 present in each reaction. IC_{50} values were calculated and are reported in Table 5 along with statistics for individual fits. (C) The ability of 1 µM BBK32 proteins to inhibit the cleavage of complement C4 by previously activated and purified C1s enzyme was evaluated *in vitro* by monitoring the conversion of the C4 α-chain to the C4 α'-chain by SDS-PAGE. C1s enzyme activity could not be detected under the conditions used for any BBK32 proteins, indicating that the BBK32 inhibitory activity is specific for C1r. C1s activity assays were performed in duplicate and a gel image from a representative experiment is shown.

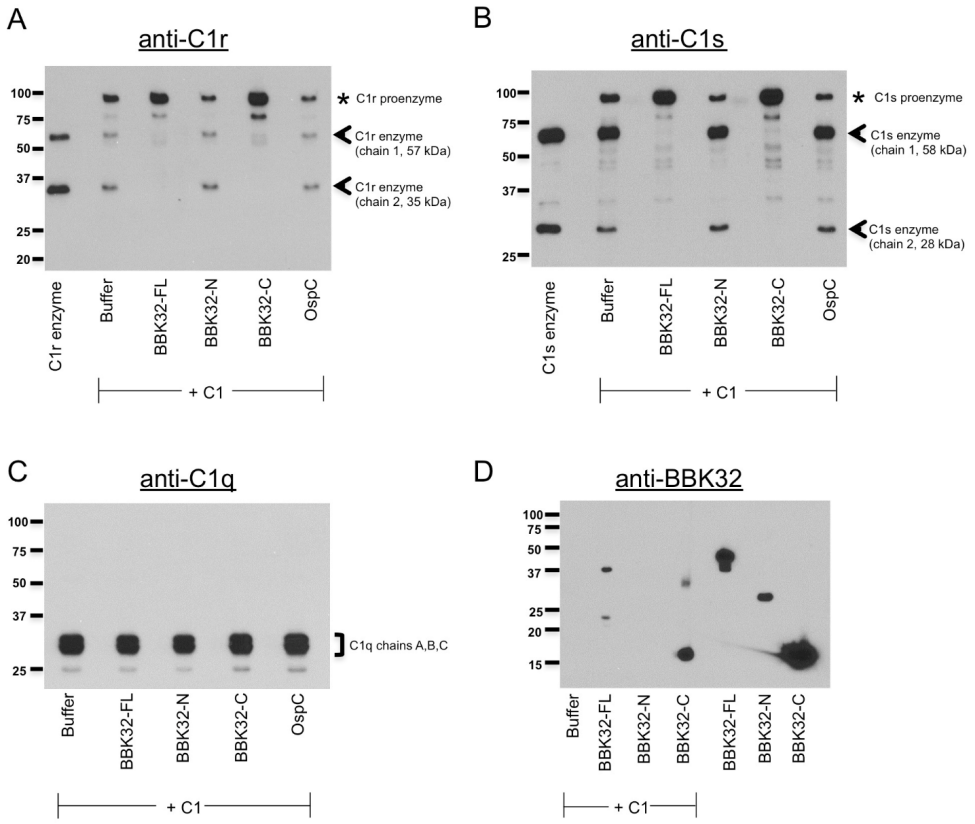

Figure 19. BBK32 inhibits the autocatalysis of C1r within the C1 complex. C1 (40 nM) was incubated at 37°C for 2 hours in the presence or absence of 5 μM BBK32 or OspC proteins. Reactions were co-immunoprecipitated using a C1q monoclonal antibody previously adsorbed to protein G beads, and bound fractions were subjected to Western immunoblot analysis. (A) Reactions were probed with C1r polyclonal antibody. Previously purified activated C1r enzyme is loaded as a reference. Activated C1r is detected in buffer only reactions as judged by the presence of C1r enzyme chain 1 (57 kDa) and chain 2 (35 kDa), denoted by arrowheads. Reactions incubated with BBK32-FL or BBK32-C lack processed C1r and contained only the 92 kDa C1r proenzyme form (denoted by an asterisk). BBK32-N and a negative control protein (OspC) reactions contain processed C1r at levels indistinguishable from the buffer only reaction. (B) Reactions were probed with a C1s polyclonal antibody and previously purified activated C1s is loaded for reference. As was observed for C1r, reactions incubated with BBK32-FL or BBK32-C lack activated C1s and contain only C1s proenzyme (asterisk). Buffer only, BBK32-N, and OspC reactions contain equal amounts of activated C1s as judged by the presence of C1s enzyme chain 1 (58 kDa) and chain 2 (28 kDa) (denoted with arrowheads). (C) Detection with C1q polyclonal antibody indicates equivalent amounts of C1q are pulled down in all reactions. (D) Detection with BBK32 polyclonal antibody demonstrates that BBK32-FL or BBK32-C but not BBK32-N are pulled down with the C1 complex. The last three rightmost lanes contain 100 ng of each form of BBK32 protein as a reference.

of C1r chain 1 (57 kDa) and C1r chain 2 (35 kDa). Interestingly, reactions containing BBK32-FL or BBK32-C contain only the proenzyme form of C1r (92 kDa). In contrast, reactions incubated with BBK32-N or an independent negative control (recombinant borrelial lipoprotein OspC) were indistinguishable from the buffer only reaction. These results demonstrate that BBK32 inhibits the autocatalysis of the C1r proenzyme. In agreement with the presence of inactivated C1r within the C1 complex, we found essentially only C1s proenzyme (86 kDa) when we probed the reactions containing BBK32-FL or BBK32-C with the C1s polyclonal antibody (**Fig. 19B**). As would be expected by the presence of activated C1r, we found cleaved C1s in the buffer only, BBK32-N, and OspC reactions. C1q was detected at identical levels in all reactions, confirming the validity of the co-IP approach (**Fig. 19C**), and importantly, BBK32-FL or BBK32-C but not BBK32-N were pulled down with the C1 complex (**Fig. 19D**). Taken together, these results show that BBK32 bound C1 is trapped in an inactive form that the autocatalytic activation of C1r is inhibited, that C1r cleavage of C1s proenzyme is blocked, and that the BBK32 inhibitory effect is specific for the C-terminal half of the protein.

B. burgdorferi binds to C1 and C1r in a BBK32-specific manner

We were interested in determining if similar activities were also observed with native BBK32 produced in *B. burgdorferi*. To test whether surface exposed BBK32 could mediate binding to components of the classical complement pathway, we incubated infectious *B. burgdorferi* parent strain and a *bbk32*::Str^R

derivative (strain JS315, grown under conditions that induce *bbk32*) with immobilized C1. Interestingly, both the parent and the *bbk32* isogenic mutant readily bound C1 and no significant difference in binding could be detected (**Fig. 20**). One plausible explanation for this result is a likely layer of functional redundancy provided by the presence of additional *B. burgdorferi* proteins capable of C1 recognition as evidenced in the overlay analysis (**Fig. 12A**). To provide a less complex outer surface environment for *B. burgdorferi*, we used strain B314, which is missing linear plasmids (lp) and thus does not synthesize many lp-encoded borrelial proteins associated with both mammalian and tick infectivity, specifically OspAB, DbpBA, and BBK32 (Sadziene et al., 1993, 1995). A shuttle vector containing *bbk32* with its native promoter (pCD100) was transformed into strain B314, along with a vector-only control (pBBE22*luc*). An equivalent amount of protein was loaded from each strain (**Fig. 21A**) and subsequent immunoblot and biotin-labeled C1 overlay analysis showed that, as expected, B314/pCD100 made detectable levels of BBK32 capable of binding C1 whereas B314/pBBE22*luc* did not (**Fig. 21B** and **C**). To determine whether natively produced BBK32 mirrored the C1/C1r-binding profile of purified recombinant BBK32, we exposed these strains to immobilized C1 and C1r. Unlike the wild-type strain, the B314 control strain (B314/pBBE22*luc*) exhibited very little binding to C1 or C1r (**Fig. 21D**). In contrast, the presence of BBK32 in B314/pCD100 transformed organisms promoted a significant enhancement of spirochete binding to both of these targets (**Fig. 21D**).

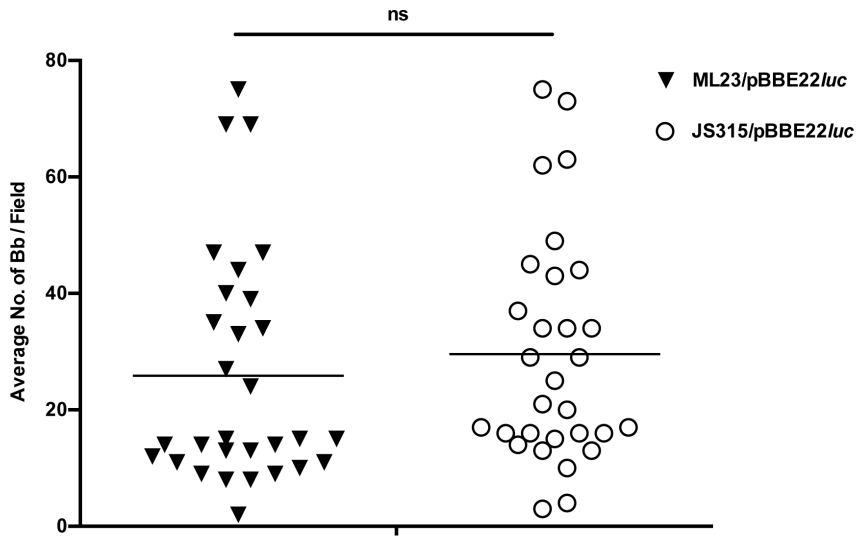

Figure 20. Infectious *B. burgdorferi* and the isogenic *bbk32* mutant bind to C1 at high levels. Strain ML23/pBBE22*luc* and the *bbk32* mutant derivative JS315/ pBBE22*luc* were incubated with immobilized C1 to assess binding. No significant difference was observed in binding for both strains tested. Each data point represents spirochetes counted within an independent field as scored by dark field microscopy (n = 30 per strain). The horizontal bar represents the mean value. Statistical significance was evaluated by use of an unpaired *t* test. ns; not significant.

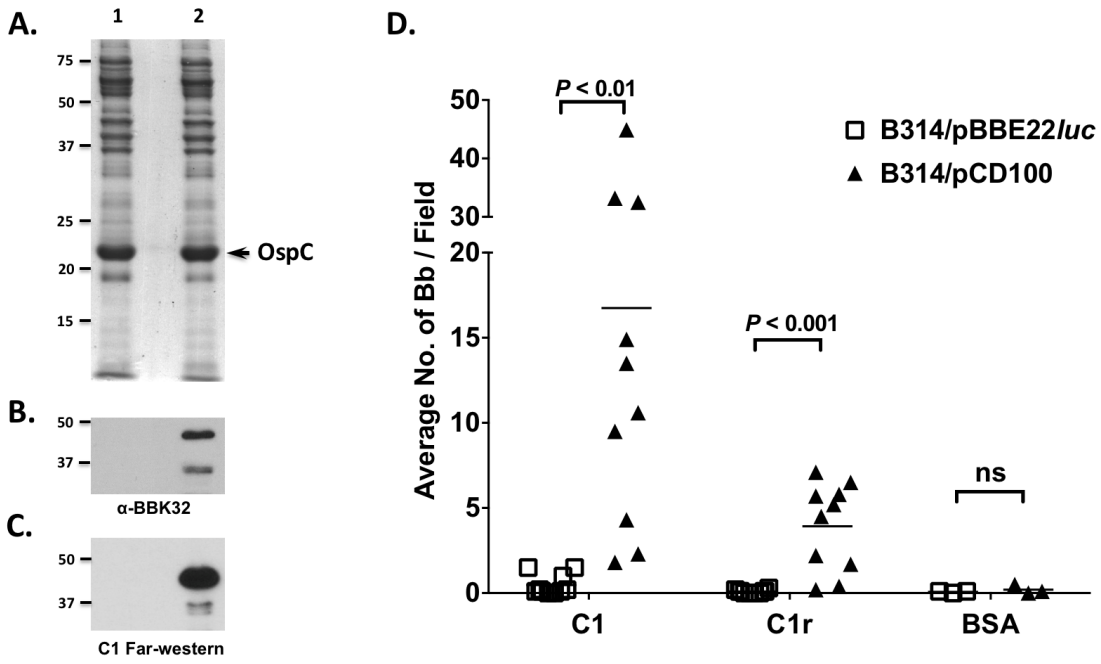

Figure 21. Surface exposed BBK32 promotes binding of a non-infectious variant B314 of *B. burgdorferi* to human C1 and C1r. Strain B314 total cell protein lysates from cells containing the vector pBBE22*luc* (lane 1) and natively expressed *bbk32* (plasmid construct pCD100; lane 2) were stained with Coomassie blue (A), immunoblotted against a monoclonal antibody specific for BBK32 (B), and subjected to Far Western overlay analysis with biotinylated C1 (C). B314/pBBE22*luc* and the *bbk32* expressing strain B314/pCD100 were incubated with immobilized C1, C1r, and BSA to assess BBK32-dependent binding to these target proteins (D). Each data point represents the average number of spirochetes present on 10 independently read fields as scored by dark field microscopy. The horizontal bar represents the mean value. Statistical significance in (D) was determined by use of a multiple *t* test followed by the Sidak-Bonferroni method to correct for multiple comparisons. All experiments were performed independently between three and five times. Significant differences are reflected in the *P* values shown. ns; not significant.

BBK32 promotes resistance to a serum sensitive B. burgdorferi strain

We were next interested if the production of BBK32 could promote resistance to serum. For this purpose, we again utilized *B. burgdorferi* strain B314, which in addition to its avirulent phenotype, is rendered serum sensitive, presumably due to its aforementioned loss of lp content (Sadziene et al., 1995). We hypothesized that the selective addition of BBK32 would make strain B314 resistant to complement dependent killing and, to assess this, tested B314/pBBE22*luc* and B314/pCD100 for their relative resistance to serum. Consistent with an anti-complement activity, the presence of natively expressed BBK32 in B314/pCD100 provided significant protection against serum based immobilization relative to strain B314/pBBE22*luc* that lacked BBK32 (**Fig. 22**). As expected, heat inactivation resulted in limited killing with either strain tested (**Fig. 22**). When conditions were used that selectively block the classical/lectin pathway but keep the alternative pathway intact (NHS + Mg-EGTA) (Dam et al., 1997) both strains exhibited equivalent resistance/sensitivity independent of BBK32 levels (**Fig. 22**). These data imply that a majority of the inactivation observed for serum-sensitive B314/pBBE22*luc* is due to the adverse effects of the classical and lectin pathways. Furthermore, these data strongly suggest that the BBK32-dependent protection observed with untreated serum is due to the ability of BBK32 to neutralize either the classical or lectin pathway. Coupled with the C1/C1r-specific binding profile (**Fig. 12, 13 and 17**), the classical pathway-specific inhibition (**Fig.**

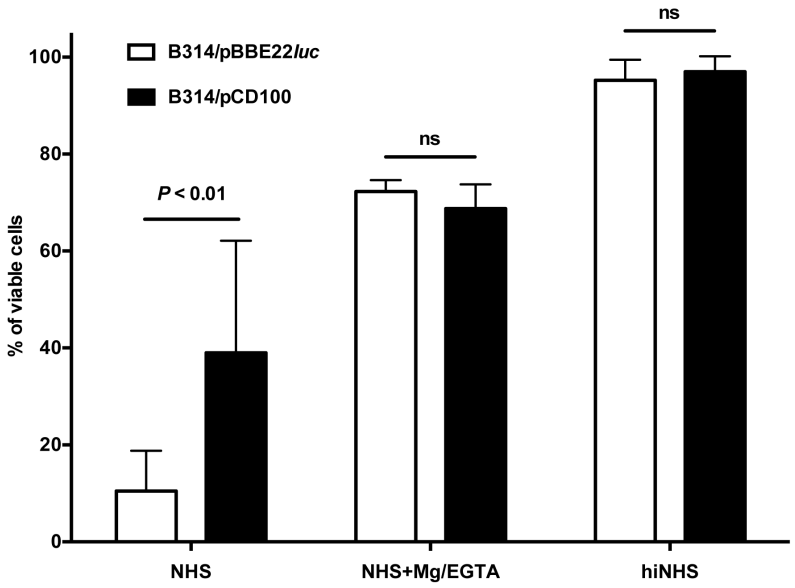

Figure 22. Production of BBK32 provides resistance to the serum sensitive strain B314. *B. burgdorferi* strain natively expressing BBK32 (B314/pCD100) is significantly more resistant to serum-based inactivation relative to the isogenic parent strain B314/pBBE22*luc*. Conditions that selectively block the classical/lectin pathway but leave the alternative pathway intact (NHS+Mg/EGTA) enhanced survival of these cells in a BBK32-independent manner. As expected, heat inactivated serum (hiNHS) ablated all complement-dependent killing for both strains. Statistical significance was determined by use of a multiple *t* test followed by the Sidak-Bonferroni method to correct for multiple comparisons and is depicted as the mean value ± the standard deviation. All experiments were performed independently between three and five times. Significant differences are reflected in the *P* values shown. ns; not significant.

14 and 15), and C1r inhibitory activity (**Fig. 17 and 18**) of recombinant BBK32 proteins along with the classical pathway-specific function of C1r (Thiel et al., 2000), the serum resistance mediated by BBK32 in this assay is best explained by BBK32-dependent inactivation of the classical pathway.

Naïve secreted IgM mediates complement killing of the non-infectious variant B314 strain of B. burgdorferi

The classical complement pathway is associated with antigen-specific antibody dependent killing. However, the NHS used in this study does not contain *B. burgdorferi* specific antibodies (data not shown), indicating that the classical pathway dependent killing of the non-infectious *B. burgdorferi* partially reflects an innate immune defense. In fact, in addition to the antigen-specific IgG, the C1 complex can be also activated by naïve IgM antibodies, which exist as a pentamer in the circulation system, and have a 1000-fold greater binding affinity to C1q when compared to IgG. (Ehrenstein and Notley, 2010; Ochsenbein and Zinkernagel, 2000). Therefore, we tested if the secreted naïve, and non-immunized IgM in NHS mediates the classical complement dependent killing of the serum-sensitive *B. burgdorferi* B314 strain. A C4b deposition ELISA assay on an IgM-coated plate showed that IgM-depleted serum retained the same level of the classical pathway activity, when compared to NHS (**Fig. 23A**). We then performed the serum sensitivity assay in NHS and IgM-depleted serum. Consistent with prior result (**Fig. 22**), B314/pBBE22*luc* that lacked BBK32 was

sensitive to NHS, and expression of *bbk32* partially restored borrelial serum resistance (**Fig. 23B**). As a comparison, when treated with IgM-depleted serum, both strains exhibited similar resistance/sensitivity independent of BBK32 levels (**Fig. 23B**), and the survival rate of both strains was recovered to the same level as when they were incubated with Mg-EGTA treated serum that compromises the classical pathway (**Fig. 22**). These data imply that naïve IgM antibodies presented in NHS activate the classical complement pathway and kill *B. burgdorferi* that lack serum resistant activity. Furthermore, these data suggest that the classical pathway plays a role in the innate immune response to control *B. burgdorferi* infection in an IgM-dependent manner.

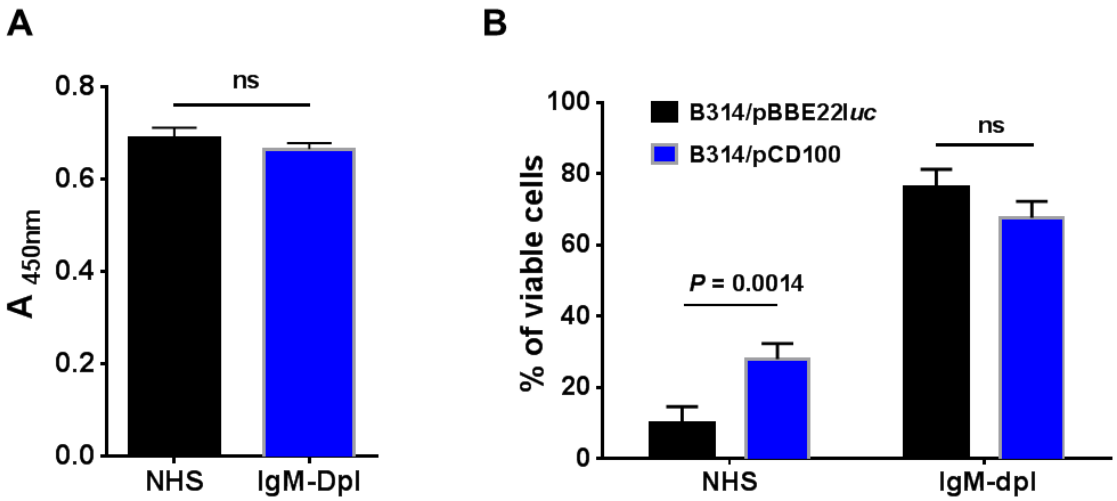

Figure 23. Naïve secreted IgM mediates complement killing of the non-infectious variant B314 strain of *B. burgdorferi*. A. The classical complement pathway is equally activated by immobilization of human IgM followed by incubation of 1% normal human serum or IgM-depleted serum in GHB^{++} buffer. Polyclonal antibodies were used to detect the deposition of the complement activation products C4b. Statistical significance was determined by use of a student *t* test and is depicted as the mean value ± the standard deviation. B. Non-infectious *B. burgdorferi* B314 strain expressing BBK32 (B314/pCD100) is significantly more resistant to serum-based killing, compared with the isogenic parent strain B314/pBBE22*luc*. Depletion of IgM from the serum enhanced survival of these cells in a BBK32-independent manner. Statistical significance was determined by use of a multiple *t* test followed by the Sidak-Bonferroni method to correct for multiple comparisons and is depicted as the mean value ± the standard deviation. All experiments were performed independently three times. Significant differences are reflected in the *P* values shown. ns; not significant.

Discussion

In order to survive the destructive forces of the human complement cascade, successful microbial pathogens have evolved a number of sophisticated evasion strategies. Although specific modes of complement recognition and inactivation are widely varied, these inhibitory mechanisms can be conceptually grouped into three forms: direct recruitment or mimicry of host regulators of complement activity such as complement factor H, enzymatic degradation of complement components by direct or indirect means, or inhibition through direct interaction with complement proteins (Lambris et al., 2008). In addition to employing each of these evasion strategies, *B. burgdorferi* takes advantage of its complex mammalian-tick lifestyle by exploiting anti-complement molecules produced by the tick salivary glands during tick feeding (de Taeye et al., 2013).

Recruitment of factor H related molecules by the borrelial CspA, CspZ, and the OspE/F proteins has been studied extensively (Kraiczy and Stevenson, 2013; de Taeye et al., 2013) and C4b-binding protein has been reported to interact with *B. burgdorferi, B. afzelii, and B. garinii* (Pietikäinen et al., 2010), while the relapsing fever spirochetes have been shown to bind factor H (McDowell et al., 2003b; Hovis et al., 2004) C4b-binding protein and C1-INH (Grosskinsky et al., 2010; Meri et al., 2006). In addition to their direct recruitment, an example of host complement regulator mimicry has also previously been reported for *B. burgdorferi* (Pausa et al., 2003). To our knowledge, the only previously reported example of a borrelial factor possessing direct and novel complement inhibition

activity is the recently described terminal complement complex inhibition function of CspA (Hallström et al., 2013; de Taeye et al., 2013). CspA along with its ability to bind factor H/factor H like-1 and plasminogen also acts a direct inhibitor of the terminal complement complex by binding complement components C7 and C9 and interfering with C5b-9 complex assembly (Hallström et al., 2013).

All three pathways of complement can be activated by *B. burgdorferi* and all result in direct complement-mediated killing of the spirochete (Hallström et al., 2013; de Taeye et al., 2013). In addition to becoming activated by immune complexes, the classical pathway has also been shown to kill *B. burgdorferi* in the absence of specific antibodies (Dam et al., 1997). The requirement for protection from complement attack for *B. burgdorferi* is evidenced by the production of a number of virulence factors (now to include BBK32) that specifically target and inactivate complement (Hallström et al., 2013; de Taeye et al., 2013). In this study we determined that the C-terminal globular region of the borrelial lipoprotein BBK32 exhibits a potent classical pathway-specific inhibitory activity that confers serum resistance to a normally serum-sensitive *B. burgdorferi* strain. While the focus of this study was on elucidating the anti-complement activity and mechanism of BBK32, our probe of lysates with C1 and C1r suggest a robust interaction between *B. burgdorferi* and the classical pathway may exist. Despite the linkage of *B. burgdorferi* to complement resistance, the small number of experimental infectivity studies employing mice deficient in key components of complement has shown a surprisingly limited role for complement in controlling *B.*

burgdorferi burden (Bockenstedt et al., 1993; Lawrenz et al., 2003; Woodman et al., 2007). Thus, it is of interest to consider potential roles for the inhibition of the classical pathway beyond protection from complement-mediated attack. For example, upon colonizing lymph tissue *B. burgdorferi* disrupts the normal formation of germinal centers (GC) (Elsner et al., 2015; Hastey et al., 2012). Lack of normal GC development ultimately results in reduced antibody titers against *B. burgdorferi* in experimental infection (Elsner et al., 2015). Local complement C4 deposition on follicular dendritic cells (FDC) is significantly reduced in *B. burgdorferi* infected lymph nodes and this is speculated to be responsible for the premature collapse of GC responses due to diminished antigen presentation by FDCs (Elsner et al., 2015). In this regard, it is of interest to determine if BBK32 mediates this lymphoid specific effect, resulting in the observed reduction in the humoral immune response to borrelial antigens. It would be of additional interest to understand the potential role of BBK32 anti-complement activity in the context of spirochetal persistence in a natural reservoir animal such as *Peromyscus leucopus* (Cook and Barbour, 2015). Studies to address some of these possibilities are currently underway.

In the current study we show that BBK32 acts directly by binding to and inhibiting C1 via a novel mechanism involving the noncovalent inhibition of C1r enzymatic activity. A model for the inhibition of C1 by BBK32 is depicted in **Fig. 24**. When C1 engages a surface via C1q binding, this information is transmitted by coordinated conformational changes within the C1 complex ultimately

triggering autocatalysis of C1r and subsequent activation of C1s (Venkatraman Girija et al., 2013). Under physiological conditions this activation is extremely rapid (Ziccardi, 1981) and controlled on the surface of host cells by the only known endogenous inhibitor of C1, C1-INH. C1-INH covalently modifies the C1r and C1s active sites and promotes their release from ligand-bound C1q (Davis et al., 2008). C1-INH is a member of the serpin family of protease inhibitors and along with inhibiting both C1r and C1s it also inactivates a number of blood proteases involved in the complement, contact, fibrinolytic, and coagulation systems (Davis et al., 2008). The broad protease-binding specificity and covalent inhibitory mechanism of C1-INH stands in stark contrast to that of BBK32, which specifically binds and inhibits C1r and had no detectable effect on the homologous C1s protease. It has been shown that serum deficient in C1r is unable to undergo classical pathway activation while retaining a fully functional lectin pathway (Thiel et al., 2000). In our studies BBK32 did not block the lectin or alternative pathway, suggesting it is unable to inhibit the primary serine proteases of these pathways (i.e., MASPs and factor D, respectively). Instead, BBK32 specifically inactivates the classical pathway by preventing the autocatalysis of C1r proenzyme and subsequent cleavage of C1s proenzyme ultimately rendering C1 entrapped in a zymogen form.

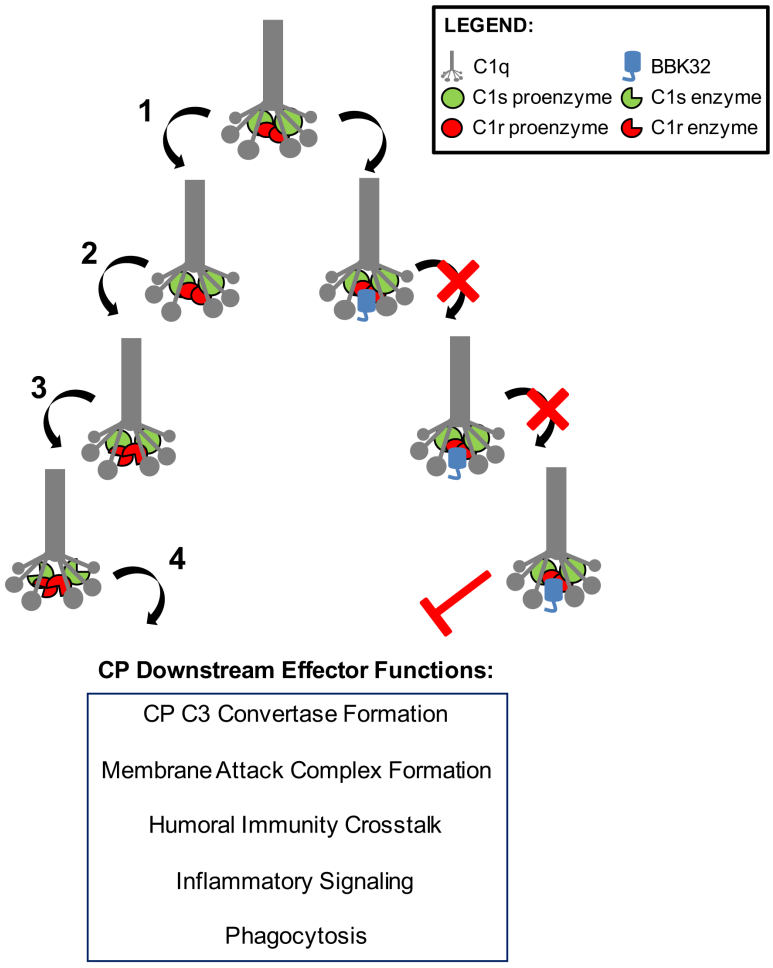

Figure 24. Model of C1 inactivation and the classical pathway inhibition by BBK32. C1 circulates as an inactive zymogen composed of one C1q molecule, two C1r proenzyme molecules, and two C1s proenzyme molecules. The pathway on the left depicts the normal uninhibited activation of C1 that proceeds by binding of the pattern recognition molecule C1q to a bacterial surface (1). C1q binding triggers the autocatalysis of the C1r proenzymes (2) that subsequently cleave and activate C1s (3). C1s then cleaves downstream the classical pathway components (4), which ultimately lead to many downstream the classical pathway effector functions. The pathway on the right side depicts the situation in the presence of the BBK32 lipoprotein. Following C1q binding to the borrelial surface the C-terminal domain of BBK32 recognizes C1 by binding directly to C1r. BBK32 blocks the autocatalytic and C1s proenzyme proteolysis functions of C1r (denoted in both instances by a red X). Thus, BBK32 effectively renders C1 in the zymogen form leading to abrogation of the classical pathway activation (denoted by a red bar).

Since its discovery as a fibronectin and glycosoaminoglycan-binding protein expressed on the surface of *B. burgdorferi* (Fischer et al., 2006; Probert and Johnson, 1998), BBK32 has been the subject of intense study (Fikrig et al., 2000; He et al., 2007; Hyde et al., 2011a; Kim et al., 2004; Lin et al., 2014; Raibaud et al., 2005; Seshu et al., 2006), including a recent report indicating a role in bloodstream survival (Caine and Coburn, 2015). In this regard, BBK32 has been shown to be critical for borrelial pathogenesis (Hyde et al., 2011a; Seshu et al., 2006), capable of exploiting host fibronectin function (Harris et al., 2014; Kim et al., 2004; Moriarty et al., 2012; Norman et al., 2008; Prabhakaran et al., 2009; Raibaud et al., 2005), shown to be involved in borrelial vascular adhesion mechanisms (Caine and Coburn, 2015; Moriarty et al., 2012; Norman et al., 2008), and in promoting joint colonization (Lin et al., 2014). Surprisingly then, *B. burgdorferi bbk32* mutant shows a limited infectivity phenotype when experimental infection is done at a high inoculum dose (i.e., 10^5) (Hyde et al., 2011a; Li et al., 2006; Seshu et al., 2006). However, this effect is restricted to high doses, as lower inoculum doses (e.g., 10^3) exhibit a significant reduction in colonization (Seshu et al., 2006) that is most apparent using *in vivo* imaging for detection (Hyde et al., 2011a). Furthermore, *bbk32* mutant strains exhibit a delay in the ability to disseminate (Hyde et al., 2011a). Nonetheless, one potential explanation for the relatively mild phenotype of the *bbk32* mutant is a layer of functional redundancy present in the spirochete. For example, several borrelial proteins besides BBK32 are now known to bind directly to fibronectin (Brissette et al., 2009c; Gaultney et

al., 2013; Hallström et al., 2010), and although much remains to be known about their specific functional activities, it is possible that these proteins could overlap with BBK32's fibronectin related functions. It seems a similar situation may exist for the BBK32 anti-complement activities described here. The presence of multiple bands in the Far Western lysate probes (**Fig 12 and Fig 16 E**) suggest that *B. burgdorferi* may express one or more "non-BBK32" proteins capable of interacting with both C1 and C1r. The existence of additional borrelial proteins able to compensate a loss of BBK32 may further explain the muted *bbk32* mutant phenotype. Determining the identity of these proteins along with evaluating their potential to interfere with the classical pathway remains an important next step.

Orthologs of BBK32 are found not only in Lyme disease spirochetes, but also in relapsing fever spirochetes where they have been divided into three groups based on phylogenetic relationships (Lewis et al., 2014). Interestingly, the Lyme disease spirochete *B. valaisiana* strain ZWU3 Ny3 was shown to possess a novel mechanism of complement inhibition independent of host complement regulator recruitment/mimicry (Schwab et al., 2013). Although a molecular mechanism has not been elucidated, the authors hypothesize that the inhibitory mechanism present in this strain of *B. valaisiana* relies on direct interaction with complement components (Schwab et al., 2013). We note that a BLASTP search of BBK32 from *B. burgdorferi* B31 reveals a gene located on linear plasmid 28 of *B. valaisiana* strain VS116 that encodes a 113 amino acids residue hypothetical protein that contains 60% identity and 72% similarity with the C-terminus of BBK32 (residues

181 to 303). While it is not known if this is a functional gene product possessing BBK32 anti-complement activity, it is intriguing that the hypothetical protein lacks the GAG and Fn-binding sequences that are the hallmark of BBK32. Nonetheless, future studies are required to understand if the complement inhibitory activity of the BBK32 C-terminal domain is conserved across borrelial species.

In the same light, it remains to be seen if BBK32 anti-complement mechanisms are found in other human pathogens that are known to have evolved similar molecular mechanisms of host interaction. For instance, BBK32 itself shares nearly identical modes of fibronectin interaction with a group of fibronectin-binding proteins from staphylococcal and streptococcal bacterial species (Bingham et al., 2008; Marjenberg et al., 2011; Meenan et al., 2007; Raibaud et al., 2005; Schwarz-Linek et al., 2003). While these Gram-positive encoded proteins lack sequence conservation with C-terminal BBK32 sequences, it may be important to assess their potential role in complement interaction. The possibility is raised that an underlying benefit to the microorganism exists in producing proteins capable of simultaneously interacting with host extracellular matrix molecules like fibronectin and components of the complement system. Such an example of a synergistic mode of interaction exists for the extracellular fibrinogen-binding (Efb) protein expressed by *Staphylococcus aureus*. A disordered N-terminal region of Efb binds directly to human fibrinogen, while a highly basic globular domain originating from the C-terminal region of the protein binds with high affinity to complement component C3 (Hammel et al., 2007; Ko et

al., 2011; Lee et al., 2004). Although each molecular interaction individually contributes to virulence, a ternary fibrinogen-Efb-C3b complex can form, encapsulating the bacteria in a 'fibrinogen-shield', which results in direct inhibition of phagocytosis (Ko et al., 2013). While the similarity of the BBK32 molecular architecture to that of staphylococcal Efb is striking, it is currently unknown how BBK32/fibronectin binding affects BBK32 anti-complement activities or if an analogous functional synergism exists. The work presented here provides the conceptual framework to explore this and related questions on complex host-pathogen interactions involving extracellular matrix and complement proteins.

Experimental procedures

Bacterial strains and plasmids

B. burgdorferi B31 strains ML23 (Labandeira-Rey et al., 2001), JS315 (ML23 *bbk32*::Str^R; (Seshu et al., 2006)), and B314 were grown in BSK-II media supplemented with 6% normal rabbit serum (Pel-Freez Biologicals, Rogers, AR) under microaerobic conditions at 37°C, 5% CO_2 atmosphere, at either a pH of 6.8 or 7.6. The serum sensitive strain B314 (kindly provided by Tom Schwan) is a non-infectious variant of strain B31 that lacks most linear plasmids (Fischer et al., 2006; Lin et al., 2014; Sadziene et al., 1993, 1995). All *B. burgdorferi* cells were enumerated by dark field microscopy.

Native *bbk32* was cloned into the shuttle vector pBBE22*luc* (Hyde et al., 2011a) using the following approach. The oligonucleotide primers BBK32Comp-

BamHI-F (5'-ACGC**GGATCC**GTACTTTGTTCACCCTCTTGATAGC-3'; BamHI site is in **bold**) and BBK32Comp-SalI-R (5'-ACGCGTCGACATATTATGTAGCCTGTTTTAAATT-3'; SalI site is underlined) were used to PCR amplify *bbk32* from strain B31 genomic DNA. The PCR-amplified product contains 213 bp of upstream sequence and 37 bp downstream from the translational start site and stop codon of the 1,062 bp *bbk32* gene, respectively. The resulting 1.3 kb fragment was cloned into plasmid pCR-Blunt-II-TOPO and transformed in Mach1-T1R *Escherichia coli* cells (F^- ∅80*lac*ZΔM15 Δ*lac*X74 *hsd*R(rK–, mK+) Δ*rec*A1398 *end*A1 *ton*A; ThermoFisher). The resulting construct was digested with BamHI and SalI and the 1.3 kb fragment subsequently cloned into BamHI and SalI cut pBBE22*luc*. The resulting construct, which contained *bbk32* expressed from its native promoter, was designated pCD100.

Transformation of strain B314 with pCD100 and pBBE22*luc* was done as previously described (Samuels, 1995). Transformants were selected for resistance to kanamycin and screened by PCR to confirm the presence of pCD100 using primers BBK32Comp-BamHI-F and BBK32Comp-SalI-R.

Proteinase K accessibility assay

B. burgdorferi strain ML23 was grown under inducing conditions (37˚C, 5% CO_2, pH 6.8) and harvested by centrifugation at 5,800 x *g*, and washed twice with PBS. The cell pellet was resuspended in 0.5 ml of either PBS alone, or PBS with proteinase K (to a final concentration of 200 μg ml^{-1}). All samples were incubated

at 20°C for 40 min. Reactions were terminated by the addition of phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 1 mM. Cells were again pelleted by centrifugation (9,000 x *g* for 10 min at 4°C), washed twice with PBS containing 1 mM PMSF, and resuspended in Laemmli sample buffer (Laemmli, 1970). Samples corresponding to 5 x 10^7 whole cell equivalents were run on SDS-PAGE gel, transferred to PVDF membranes and probed with biotinylated C1 in a Far Western analysis or immunoblotted with a monoclonal antibody to borrelial FlaB (Affinity BioReagents, Inc.), as described below.

Proteins

DNA fragments encoding residues 21 to 205 or 206 to 354 of BBK32 (*B. burgdorferi* B31 strain) were PCR-amplified from pQE30-BBK32-FL plasmid DNA (Kim et al., 2004) using oligonucleotide primers that appended BamHI and NotI sites at the 5' and 3' ends, respectively. Restriction digested DNA fragments were then sub-cloned into the pT7HMT vector (Geisbrecht et al., 2006). Sequence confirmed plasmids were transformed into *E. coli* strain BL21(DE3) for protein production.

Expression and purification of recombinant BBK32-FL was performed as previously described (Kim et al., 2004). Recombinant BBK32-N and BBK32-C were overexpressed by inoculating 1 liter of Terrific Broth with 10 ml of an overnight BL21(DE3) at 37°C, induced with 1 mM isopropyl-D-thiogalactopyranoside upon reaching an optical density at 600 nm of 0.6-0.8,

shifted to 18°C shaking, and allowed to express overnight. Overnight cultures were harvested by centrifugation at 5,000 x *g* for 10 min and resuspended in Ni-NTA-binding buffer (20 mM Tris (pH 8.0), 500 mM NaCl, 10 mM Imidazole (pH 8.0)) and lysed by microfluidization. A clarified cell extract was obtained by centrifugation at 25,000 x *g* for 35 min and the supernatant was applied to a nickel agarose (Gold Bio) column previously equilibrated in Ni-NTA buffer, washed with 5 column volumes (CV) of Ni-NTA-binding buffer, and eluted with 2 CV's of Ni-NTA elution buffer (20 mM Tris (pH 8.0), 500 mM NaCl, 500 mM imidazole (pH 8.0)). The HIS-myc affinity tag was removed by enzymatic digestion with tobacco etch virus (TEV) protease in the presence of 5 mM β-mercaptoethanol at room temperature for 2 h. The digestion reaction was allowed to continue overnight at 4°C while being dialyzed against Ni-NTA-binding buffer. The TEV digested sample was then incubated with a nickel agarose column and the flow through fraction was collected and subjected to gel filtration chromatography using a HiLoad Superdex 200 PG column (GE Healthcare) equilibrated in 20 mM Tris (8.0), 200 mM NaCl. Peaks were analyzed by SDS-PAGE and fractions corresponding to BBK32 proteins were pooled and concentrated using Amicon centrifugal filters (EMD Millipore), aliquoted, and stored at -80°C until use.

Purified C1, C1r enzyme, C1q, C1s enzyme, C1s proenzyme, and C4 were obtained from Complement Technology (Tyler, TX). Human C1 or C1r proteins were biotinylated using EZ-link Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific) at the molar ratio suggested by the manufacturer. The labeling reaction was

quenched by the addition of Tris HCl pH 7.6 to a final concentration of 20 mM. The sample was diluted in protein-binding buffer (100 mM NaCl, 20 mM Tris pH 7.6, 1 mM EDTA, 10% glycerol, 0.2% Tween-20, 2% non-fat milk) (Wu et al., 2007) and used below in the Far-Western assay.

Far-Western immunoblotting and conventional immunoblotting

For Far Western analyses, *B. burgdorferi* protein lysates were resolved by SDS–PAGE (Laemmli, 1970) and gels were transferred to PVDF membranes as described (Weening et al., 2008). The membrane was blocked in 10% non-fat milk, washed with PBS, 0.2% Tween-20, and then 20 µg biotinylated C1 or C1r (both from CompTech), at 1 µg/ml in protein binding buffer (Wu et al., 2007), was incubated with the membrane overnight at 4°C. The membrane was washed extensively in PBS, 0.2% Tween-20, and then incubated with Vectastain solution (Vectastain Elite ABC Kit, Vector Laboratories), as instructed by the manufacturer, to enhance the signal. The membrane was then washed and developed using the Western Lightning Chemiluminescent Reagent plus system (Perkin Elmer, Waltham, MA, USA).

Conventional immunoblotting was done as previously described [94]. Production of BBK32 in B314/pCD100 was evaluated using either a monoclonal antibody to BBK32 (a generous gift from Seppo Meri, University of Helsinki) diluted to 1:4000 or, in the case of the co-immunoprecipitations (see below), a polyclonal antibody against BBK32 diluted 1:1500. Immunoblots to detect the

endoflagellar antigen FlaB were done using a monoclonal to *B. burgdorferi* FlaB (Affinity BioReagents) diluted 1:20,000. Appropriate anti-rabbit Ig or anti-mouse Ig HRP conjugates (Life Technologies) were diluted 1:5000 and used to detect primary antibodies on the PVDF membranes. Immune complexes were detected using the Western Lightning Chemiluminescent Reagent plus system (Perkin Elmer, Waltham, MA, USA).

SPR

Direct binding of C1 and subunits of the C1 complex to BBK32 was assessed by SPR using a Biacore 3000 instrument (GE Healthcare) at 25°C. HBS-T-Ca^{2+} (20 mM HEPES (pH 7.3), 140 mM NaCl, 0.005% (v/v) Tween 20, 5 mM $CaCl_2$) was used as the running buffer and a flowrate of 10 µl min^{-1} was used in all experiments. A BBK32 biosensor was created by immobilizing recombinant BBK32 proteins on separate flowcells of a C1 sensor chip (GE Healthcare). In all cases immobilization was achieved using standard amine coupling chemistry by activating the flowcell surface for 7 min at 5 µl min^{-1} with an equal volume mixture of 0.1 M N-hydroxysuccinimide and 0.4 M ethyl(dimethylaminopropyl) carbodiimide. Next, BBK32-FL at 20 µg ml^{-1} in 10 mM sodium acetate (pH 4.0), BBK32-N at 25 µg ml^{-1} in 10 mM sodium acetate (pH 4.5), or BBK32-C at 5 µg ml^{-1} in 10 mM sodium acetate (pH 5.5) were injected and allowed to react until the desired surface density was reached. Finally, 1 M ethanolamine (pH 8.5) was injected for 7 min at 5 µl min^{-1} to quench remaining reactive groups. A reference

flowcell was generated by activating the surface followed by immediate quenching. Final immobilization densities reported in resonance units (RU) were as follows: BBK32-FL (165 RU), BBK32-N (150 RU), and BBK32-C (240 RU). All solution phase analytes were exchanged into running buffer just prior to injection.

Purified C1 was injected as a twofold concentration series in triplicate consisting of 0.20, 0.39, 0.78, 1.6, 3.1, 6.3, 13, 25, 50, and 100 nM for 3 min followed by 5 min of dissociation. The surface was regenerated to baseline by injecting HBS-T-EGTA (20 mM HEPES (pH 7.3), 140 mM NaCl, 0.005% Tween-20, and 10 mM EGTA) for 1 min followed by a 30 s injection of a solution containing 0.1 M glycine (pH 2.2) and 2.5 M NaCl. Direct interaction of C1r enzyme with BBK32 proteins was assessed using an identical protocol to C1 except for a concentration series consisting of 0.39, 0.78, 1.6, 3.1, 6.3, 13, 25, 50, 100, and 200 nM was used and the injection time was increased to 5 min followed by 10 min of dissociation. Binding of C1r enzyme to active BBK32 proteins was assessed on a second C1 biosensor with different coupling densities BBK32-FL (80 RU) and BBK32-C (330 RU). A separate concentration series consisting of 2.0, 3.9, 7.8, 16, 31, 63, 125, 250, 500, and 1000 nM was injected in duplicate for 5 min followed by 15 min of dissociation. Kinetic analysis was performed for each set of sensorgrams resulting from C1 or C1r enzyme injections using BIAevaluation software 4.1.1 (GE Healthcare) using a 1:1 (Langmuir) binding model and fitting R_{max} locally. To determine the effect of calcium on the interaction of BBK32, a single concentration of C1 (50 nM) or C1r enzyme (200 nM) was

injected in triplicate in a running buffer of HBS-T-Ca^{2+} or HBS-T-EGTA. To evaluate relative binding of individual C1 complex components to BBK32, triplicate injections were performed at a fixed 50 nM concentration in HBS-T- Ca^{2+} running buffer. Response was corrected for the molecular weight of each component (C1 = 790 kDa, C1q = 410 kDa, C1r enzyme = 92 kDa, C1s proenzyme and C1s enzyme = 86 kDa).

Complement Inhibition ELISA

To delineate the effect of BBK32 proteins on the classical, lectin, and alternative pathway, we adopted an ELISA based assay previously described (Roos et al., 2003). Costar EIA/RIA plates (Fisher Scientific) were incubated overnight at room temperature with 3 µg ml^{-1} human IgM (the classical pathway initiator) (Athens Research & Technology), 25 µg ml^{-1} *Salmonella enteriditis* LPS (the alternative pathway initiator) (Sigma Aldrich), or 20 µg ml^{-1} of mannan from *Saccharomyces cerevisiae* (the lectin pathway initiator) (Sigma Aldrich) in a coating buffer consisting of 100 mM Na_2CO_3/$NaHCO_3$ (pH 9.6). All subsequent steps were preceded by three consecutive washes with TBS-T buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 0.05% (v/v) Triton X-100) and all reaction volumes were 100 µl. Plates were blocked in PBS-T-BSA (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 1% (w/v) bovine serum albumin, and 0.05% (v/v) Tween-20) for 1 h. Reactions consisted of pooled complement human serum (Innovative Research), at 1% (the classical and lectin pathway) or 20% (the

alternative pathway) final concentration, various concentrations of BBK32 proteins, and classical/lectin pathway buffer (20 mM HEPES (pH 7.3), 0.1% (w/v) gelatin, 140 mM NaCl, 2 mM $CaCl_2$, 0.5 mM $MgCl_2$) or alternative pathway buffer (20 mM HEPES (pH 7.5), 0.1% (w/v) gelatin, 140 mM NaCl, 5 mM $MgCl_2$, 10 mM EGTA). Serum/BBK32 mixtures were then added to wells and incubated at 37°C for 1 h. Downstream complement activation was measured by detecting C3b deposition using a 1:300 dilution of an anti-C3d monoclonal antibody (030-08, Santa Cruz Biotechnology) or 1:300 dilution of an anti-C4d monoclonal antibody (C4-1, Cell Sciences) incubated at room temperature for 1 h, and subsequent 1 h room temperature incubation with a 1:5000 dilution of goat anti-mouse HRP secondary antibody (Thermo Scientific). HRP-labeled antibody was detected using 1-step Ultra TMB (Thermo Scientific) for 10 min. The reaction was stopped by addition of 2 M sulfuric acid and the absorbance was measured at 450 nm using a VersaMax microplate reader (Molecular Devices). Wells containing serum only or where serum was replaced with buffer were treated as 100% and 0% signal, respectively. All experiments were performed a minimum of three times. IC_{50} values were evaluated by variable slope four-parameter nonlinear regression analysis performed using GraphPad Prism 5.0.

Hemolytic Assays

Inhibition of classical pathway-mediated hemolysis by BBK32 proteins was assessed using a modified classical pathway hemolytic assay (CP50). Sheep

erythrocytes (5 x 10^8 cells ml^{-1}) sensitized with human IgM (Complement Tech) were centrifuged at 500 x *g* at 4°C for 3 min and resuspended in GHB^{++} buffer (20 mM HEPES (pH 7.3), 140 mM NaCl, 0.1% gelatin (w/v), 0.15 mM $CaCl_2$, and 0.5 mM $MgCl_2$). Final volumes were 100 µl and reactions began by mixing 35 µl GHB^{++} with 20 µl of BBK32 proteins previously diluted into GHB^{++} at various concentrations, followed by 20 µl NHS (1% v/v) final concentration, and 25 µl sheep erythrocytes sensitized with human IgM. Reactions were incubated at 37°C for 1 h with intermittent shaking and clarified by centrifugation at 1000 x *g* for 3 min. 50 µl of each reaction were transferred to a 96-well flat-bottom half-area microplate and absorbance was measured at 541 nm using a VersaMax microplate reader (Molecular Devices). A well containing no BBK32 was considered as 100% lysis and background absorbance was measured by replacing NHS with buffer. Percent lysis was calculated by subtracting background readings from each well and comparing each reading to 100% controls.

Inhibition of alternative pathway-mediated hemolysis by BBK32 proteins was assessed using a modified alternative pathway hemolytic assay (APH50). Rabbit erythrocytes (Complement Tech) at 5 x 10^8 cells ml^{-1} were washed by centrifugation at 500 x *g* at 4°C for 3 min and resuspension in GHBS° buffer (20 mM HEPES (pH 7.5), 140 mM NaCl and 0.1% gelatin (w/v)). Reactions began by diluting 5 µl of 0.1M $MgCl_2$-EGTA into 30 µl GHBS°, followed by 20 µl of BBK32 proteins, followed by 20 µl of NHS, and finally 25 µl rabbit erythrocytes. Reactions

were allowed to incubate at 37°C for 30 min with intermittent agitation, clarified, and diluted 1:10 in a 96-well plate. Absorbance was measured at 412 nm and % lysis was computed as described for the CP50 assay. All experiments were repeated between two and four times. IC_{50} values were evaluated by variable slope four-parameter nonlinear regression analysis performed using GraphPad Prism 5.0.

C1r/C1s Enzyme Activity Assays

The ability of BBK32 proteins to inhibit the enzymatic cleavage of C1s proenzyme by C1r enzyme *in vitro* was performed as follows. Reactions were carried out in HBS-Ca^{2+} (20 mM HEPES (pH 7.3), 140 mM NaCl, 5 mM $CaCl_2$). Reaction volumes were 10 µl and consisted of 5 µl BBK32 protein previously diluted into water, 1 µl C1s proenzyme (1 µg μL^{-1}), 1.5 µl C1r enzyme (333 nM), and 2.5 µl of 4x HBS-Ca^{2+}. Following overnight incubation at 37°C, each reaction was mixed with 5 µl reducing SDS-PAGE Laemmli sample buffer, boiled for 5 min, and 7.5 µl of each reaction were separated on a 10% Tris-tricine SDS-PAGE gel. Following Coomassie blue staining, digital images of destained gels were captured using a FluorChem M imaging system (ProteinSimple). Densitometry was performed using AlphaView SA 3.4.0 software (ProteinSimple) and the normalized peak height of the band corresponding to C1s proenzyme was plotted against the concentration of BBK32 present in each reaction. IC_{50} values were evaluated with GraphPad Prism 5.0 using four parameter variable slope nonlinear

regression analysis and constraining the top and bottom values to 100 and 0 respectively. All experiments were repeated between two and four times.

The ability of BBK32 proteins to inhibit the enzymatic cleavage of C4 by C1s enzyme *in vitro* was performed as follows. Reaction volumes were 10 µl and consisted of 2.5 µl C4 (1 mg ml^{-1}), 1 µl C1s enzyme (1 mg ml^{-1}), 1.5 µl PBS, and 5 µl of BBK32 proteins previously diluted into PBS. Reactions were incubated at 37°C for 30 min and stopped by the addition of 5 µl SDS-PAGE reducing buffer, boiled for 5 min, and evaluated by SDS-PAGE.

C1q Co-immunoprecipitation

Monoclonal antibodies to human C1q were captured by Protein G beads (Thermo Scientific) at room-temperature for 2 h as instructed by the manufacturer. Purified C1 complex (40 nM) was incubated at room temperature for 2 h with either 5 µM BBK32-FL, BBK32-N, BBK32-C, or full-length recombinant OspC. All reactions were performed in $HEPES^{++}$ buffer (20 mM HEPES, 140 mM NaCl, 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$, pH 7.3). Subsequently, C1, pre-incubated with borrelial recombinant proteins, was added to Protein G beads pre-loaded with C1q monoclonal antibodies and incubated at 37˚C for 2 hr. The beads were then washed 5 times with $HEPES^{++}$ buffer containing 0.2% Tween-20, followed by the addition of 50 µl Laemmli sample buffer. Samples were then boiled for 10 min, separated by SDS-PAGE, transferred to PVDF membranes, and subjected to Western immunoblot analysis. The components of the C1 complex or BBK32 were

detected with polyclonal goat anti-human C1q (diluted 1:5000), goat anti-human C1r (diluted 1:3000), sheep anti-human C1s (diluted 1:3000), or rabbit polyclonal antibody to BBK32 (diluted 1:1500) followed by incubation with either HRP-conjugated TrueBlot (Rockland Antibodies) directed against goat or sheep immunoglobulin (diluted 1:2000) (for the C1 proteins) or goat anti-rabbit Ig/HRP (Life Technologies) diluted 1:5000 (for BBK32). The membranes were washed extensively in PBS, 0.2% Tween-20, and developed using the Western Lightning Chemiluminescent Reagent plus system (Perkin Elmer, Waltham, MA, USA).

***B. burgdorferi* whole cell adherence assays**

The *B. burgdorferi* adherence assay was done as previously described with slight modifications (Zhi et al., 2015). Briefly, poly-D-lysine pre-coated coverslips (Corning Biocoat) were coated with 1 μg human C1 (Comptech), C1r (Comptech), or BSA respectively and incubated at 4°C overnight. The coverslips were washed thoroughly in PBS to remove excess unbound proteins. The coverslips were then blocked with 3% BSA at room temperature for 1 hr. *B. burgdorferi* strains ML23/pBBE22*luc* and JS315/pBBE22*luc* were grown to mid-logarithmic phase at 37°C, 5% CO_2, pH 6.8 to induce expression of *bbk32*. Strains B314/pBBE22*luc* and B314/pCD100 were grown to mid-logarithmic phase at 32°C, 1% CO_2, pH 7.6 since the production of BBK32 in B314/pCD100 was high independent of growth condition. All *B. burgdorferi* strains were subsequently diluted to 10^7 organisms/ml in BSK-II medium without serum. The resulting *B. burgdorferi* samples, in 0.1 ml

volumes, were applied onto the coverslips and incubated for 2 hr at 32 °C. Unbound bacteria were removed from the coverslips by gentle washing with PBS; this wash step was repeated 7 times. The coverslips were applied to a glass slide and attached spirochetes were counted by dark field microscopy.

Serum complement sensitivity assay

Complement sensitivity assays were performed as previously described (Dam et al., 1997; Kurtenbach et al., 1998). Briefly, *B. burgdorferi strains* were grown to exponential phase at 32°C, 1% CO2, pH 7.6, and 10^6 cells suspension in 80 µl of BSK-II medium was added to 20 µl of normal human serum (NHS) or 10 mM Mg/EGTA treated NHS, or IgM depleted human serum, to give a final volume of 100 µl with 20% serum. The samples were placed in microtiter plates and the suspensions were sealed and incubated at 32 °C for 2 h. Heat-inactivated normal human serum (hiNHS) was used as a control. After incubation, *B. burgdorferi* suspensions were scored under dark field microscope and the percentage of viable *B. burgdorferi* cells were calculated from randomly chosen fields and based on immobilization, loss of cell envelope integrity, and overt lysis.

IgM depletion

1 ml NHS was incubated with 100 µl anti-human IgM (µ-chain specific) agarose (Sigma) at 4°C for 1 hour. Then the agarose was gently centrifuged at

300xg for 3 minutes, the serum supernatant collected, and directly used for subsequent C4b deposition assay and serum sensitivity assays.

CHAPTER IV

CONCLUSIONS AND DISCUSSION

B. burgdorferi surface lipoproteins are crucial for borrelial pathogenesis

The causative agent of Lyme disease, *Borrelia burgdorferi*, is a unique pathogenic spirochetal bacterium. *Borrelia* species are organized as a Gram-negative like bacterium due to the existence of a double membrane envelope structure (Samuels, 2010). The composition of the borrelial outer-membrane is distinct from other Gram-negative bacteria in that it lacks lipopolysaccharides (LPS) (Takayama et al., 1987). Instead, *Borrelia* species produce abundant lipoproteins that are displayed on their outer surface and assist in the survival, colonization, and dissemination within the hosts they infect (Garcia et al., 2016; Hallström et al., 2013; Kenedy et al., 2009; Radolf et al., 2012; Seshu et al., 2006; Tilly et al., 2006; Weening et al., 2008; Zhi et al., 2015). During the transmission between the arthropod vector and vertebrate host, the surface protein profile of *Borrelia burgdorferi* changes dramatically in a manner that is largely RpoS-dependent (Burtnick et al., 2007; Caimano et al., 2004; Hubner et al., 2001; Lybecker and Samuels, 2007; Yang et al., 2000). Indeed, some of the up-regulated lipoproteins are important to the pathogenic potential of *B. burgdorferi* as they affect different aspects of the infectivity process, including immune evasion, colonization, and trafficking in blood vessels (Coburn et al., 2013; Garcia et al., 2016; Hallström et al., 2013; Kenedy et al., 2009, 2012; Moriarty et al., 2008;

Pal et al., 2004a; Seshu et al., 2006; Tilly et al., 2006, 2007; Weening et al., 2008; Zhang and Norris, 1998; Zhi et al., 2015). Specifically, BBK32 binds to fibronectin and GAGs through its N-terminal domain, and mediates borrelial adhesion as well as trafficking in blood vessels, whereas its C-terminal domain blocks the activation of classical complement pathway (Garcia et al., 2016; Lin et al., 2014; Moriarty et al., 2008; Seshu et al., 2006). DbpA/B binds to decorin and the loss of *dbpA* leads to a significantly attenuated infectivity (Weening et al., 2008). In addition, *B. burgdorferi* VlsE provides antigenic variation to avoid immune-recognition by the mammalian hosts (Zhang and Norris, 1998), while CspA, BBK32, and other proteins provide serum-resistance to complement-dependent killing (Garcia et al., 2016; Hallström et al., 2013; Kenedy and Akins, 2011; Kenedy et al., 2009). These studies have provided insight into mechanisms utilized by *B. burgforferi* to evade the host immune system, disseminate, and interact with different components enriched within the colonized tissues. In spite of these knowns, there are many genes encoding putative lipoproteins that have no known function(s). Many of these genes are regulated by RpoS as well, suggesting that they also play a role during mammalian infection. As such, it is possible that the function of some of these lipoproteins may provide additional clarity into our understanding of the pathogenesis of *B. burgdorferi*.

In Chapter II, I first focused on a previously uncharacterized lipoprotein-encoding gene *bba33,* which is up-regulated in both feeding ticks and mammalian conditions in a RpoS-dependent manner (Boardman et al., 2008; Caimano et al.,

2007; Kumar et al., 2010). I then showed that the loss of *bba33* renders *B. burgdorferi* essentially non-infectious, as determined by the clearance of the *bba33* mutant at early stages of experimental infection (Zhi et al., 2015). This observation suggests that BBA33 is essential for borrelial infection and possibly involved in initial colonization or survival in mammalian hosts. Using a phage display experiment, we found that BBA33 specifically targets a peptide sequence that matches to the collagen VI triple-helix region. In an ELISA based assay, I then determined that BBA33 binds type IV and type VI collagens in a dose-dependent manner. In addition, synthesis of BBA33 on the surface of *B. burgdorferi* mediates attachment to collagen VI, suggesting that BBA33 functions as an adhesin and promotes borrelial colonization within collagen enriched tissues (Zhi et al., 2015).

The *bbk32* gene of *B. burgdorferi* encodes a surface lipoprotein that is up-regulated in both feeding ticks and following experimental infection in mice. BBK32 binds to fibronectin and GAGs, mediates borrelial trafficking in blood vessels (Fischer et al., 2006; Kim et al., 2004; Moriarty et al., 2012; Norman et al., 2008; Probert and Johnson, 1998; Seshu et al., 2006), and is required for optimal infection of *B. burgdorferi* (Hyde et al., 2011a; Seshu et al., 2006). In Chapter III, we identified a novel function for BBK32; specifically, that BBK32 binds to the complement C1 complex, and inhibits the activation of the classical complement pathway by specifically blocking the activation of the C1r pro-enzyme. Subsequently, we determined that the production of BBK32 in an avirulent *B. burgdorferi* B31 isolate resulted in binding to both C1 and C1r, and provided partial

resistance to classical pathway-dependent complement killing (Garcia et al., 2016).

BBA33: An essential collagen-binding protein

The interaction of *B. burgdorferi* and collagens is presumed to be a critical process during infection (Barthold et al., 1991). After the bite by infected ticks, *B. burgdorferi* is injected into the dermal layer of the skin and is deposited within the host extracellular matrix (ECM), which is enriched for collagens and other structural components (Cabello et al., 2007; Radolf et al., 2012). The observations that BBA33 promotes binding of *B. burgdorferi* to collagen VI and that *bba33* mutant is completely non-infectious suggests that this interaction is a key colonization event during the early stages of mammalian infection (Zhi et al., 2015).

The complete non-infectious phenotype of the *bba33* mutant is striking. Multiple borrelial lipoproteins (BBK32, DbpA/B, ErpX, etc) bind ECM components such as fibronectin (BBK32), laminin (ErpX), and decorin (DbpA/B) (Brissette et al., 2009a; Kenedy et al., 2012; Probert and Johnson, 1998; Weening et al., 2008). Nevertheless, several mutants of *B. burgdorferi* that lose a single adhesin, such as, Rev A (binds Fn), RevB(binds Fn), Bgp (binds GAGs), and ErpX (binds Laminin), do not exhibit a dramatically attenuated infectivity phenotype (Coburn et al., 2013). One hypothesis for this observation is that some of the binding activities for these putative adhesins may be partially overlapping and redundant.

The non-infectious phenotype of the *bba33* mutant implies that no other *B. burgdorferi* proteins can compensate for BBA33 or that such a homologue is not expressed under our experimental conditions.

Based on results obtained through the use of a peptide phage display library, we found that BBA33 binds to both type IV and type VI collagens. Type IV collagen is mainly found in the skin while type VI is enriched in skeletal muscle, the papillary dermis immediately below the dermal-epidermal junction, and around blood vessels (Watson et al., 2001). Indeed, some of the first micrographs of *B. burgdorferi* from infected tissues showed *B. burgdorferi* preferentially colonize these type IV and VI collagen enriched tissues (Barthold et al., 1991; Cadavid et al., 2003; Barthold et al., 2006; Cabello et al., 2007). Despite the known interaction between *B. burgdorferi* and collagen, no collagen adhesin has yet to be identified until our identification and characterization of BBA33 in this context (Zhi et al., 2015). New possible explanations as to why *bba33* is crucial for borrelial infectivity include the following: (1), the interaction of BBA33 and collagens may facilitate the initial colonization of *B. burgdorferi* under skin, which is an essential step for *B. burgdorferi* to invade the host tissues by binding and breaking down ECM components; (2), this interaction may “cloak” *B. burgdorferi* and hide it from the host immune response. Accordingly, the loss of BBA33 may expose the bacteria to immune system at the early stage and lead to the clearance; and/or (3), the BBA33-mediated interaction of *B. burgdorferi* with collagens may promote spirochetal colonization and penetration of blood vessels

under the skin, which would promote further dissemination through the blood circulation system. To test the first hypothesis, one could ask whether *B. burgdorferi* adheres to collagens in a dermal fibroblast model. Dermal fibroblasts are major collagen-producing cells in skin (Olsen et al., 1989). *B. burgdorferi* recognizes *in vitro* cultivated primary dermal fibroblasts (Georgilis et al., 1992; Klempner et al., 1993; Wu et al., 2011); however, no specific adhesin has been linked to this interaction. If the *bba33* mutant exhibits decreased binding to dermal fibroblasts relative to the parent and complement strains, then this would imply that BBA33 is needed for binding to these cell types. Purified collagen type VI (or peptide derivative thereof) could be used to test whether they can competitively inhibit this binding. In addition, *B. burgdorferi* is able to invade fibroblasts to prevent the killing by antibiotics and blood cells (Chmielewski and Tylewska-Wierzbanowska, 2010; Georgilis et al., 1992; Wu et al., 2011). Using the *bba33* mutant, we could ask whether invasion requires BBA33.

To test the third hypothesis that *B. burgdorferi* colonizes around blood vessels in a BBA33 dependent manner, an *in vivo* imaging system such as intravital confocal microscopy (IVM) could be used to observe the behavior of *bba33* mutant under the skin and around blood vessels (Moriarty et al., 2008). IVM has been used extensively to monitor and quantify the motility, adhesion, and trafficking of *B. burgdorferi* in live mouse tissues and blood vessels (Moriarty et al., 2008, 2012; Norman et al., 2008). One can observe the interactions of *B. burgdorferi* with host cells and tissues as well as the dynamics of these

interactions. To take the advantage of this technology, we could monitor if the *bba33* mutant is able to colonize tissues around the blood vessels under IVM and whether this interaction benefits borrelial transmission through blood vessel walls. In summary, collagens are ubiquitous in mammalian tissues that *B. burgdorferi* is known to align with during infection (Barthold et al., 1991; Zambrano et al., 2004). The interaction between collagens and *B. burgdorferi* may directly contribute to the colonization, tissue tropism, and dissemination of this bacteria. Therefore, characterization of the BBA33-collagen interaction may provide important mechanistic insight into borrelial pathogenesis.

In addition to collagen binding, the *in vivo* imaging data showed that the ∆*bba33* mutant was cleared early during infection, before dissemination begins, suggesting that BBA33 may assist in the evasion of the innate immune response. It is intriguing that the complement protein C1q contains a collagen-like triple-helical motif (Kishore and Reid, 2000), which is similar to the binding ligand of BBA33 on collagen VI. The interaction of bacterial collagen-binding protein with C1q is not unprecedented and may play a role in evading the innate and adaptive immune response. For instance, *Staphylococcus aureus* collagen-binding protein Cna binds C1q and, in doing so, inhibits the activation of the classical pathway (Kang et al., 2013). Although we found that BBA33 did not inhibit the activation of the classical pathway in an IgM dependent C4b deposition and a hemolysis assay (data not shown), the interaction of BBA33 and C1q may leads to the suppression of other innate immune response mediated by C1q.

A number of studies indicate that C1q binds to different cell types and triggers a variety of cellular response, including enhanced microbial killing by phagocytosis, induction of chemotaxis, and stimulation of oxidative metabolism (Eggleton et al., 2000). Both the C1q globular head and collagen-like tail are possible ligands for cell surface C1q receptors and binding proteins (Eggleton et al., 1998). Therefore, BBA33 may mimic or compete with the host C1q receptors and interfere with the innate immune response if it recognizes the collagen-like region of C1q. The collagen-like region of C1q is recognized by the α-2-macroglobulin receptor (also known as CD91), CD18, calreticulin and CR1 (Eggleton et al., 2000; Nayak et al., 2010), each of which triggers different responses. For instance, CD91 enhances C1q-mediated phagocytosis of apoptotic cells by macrophages and epithelial cells (Duus et al., 2010; Monks et al., 2005), while CD18 mediates C1q-dependent neutrophil superoxide production (Goodman et al., 1995). BBA33 could promote borrelial entry into epithelial cells by binding to C1q; or it may interfere with the neutrophil superoxide production and promote borrelial evasion of neutrophil dependent killing as a result of competing with CD18 (Goodman et al., 1995). Based on these possibilities, *B. burgdorferi* could benefit from recruiting C1q on its surface during the early stage of infection.

Taken together, BBA33 is an essential lipoprotein for *B. burgdorferi* to establish successful mammalian infection. In the work presented herein, we show that BBA33 promotes borrelial binding to collagen type VI *in vitro* and may

promote borrelial initial colonization and/or dissemination *in vivo*. In addition, it is worth noting that BBA33 may be involved in evading the host immune response as well, potentially by mitigating the innate immune response.

BBK32: A novel complement regulator

BBK32 is an extensively studied borrelial lipoprotein that is involved in borrelial pathogenesis (Hyde et al., 2011a; Seshu et al., 2006). Previous studies have focused on the role of BBK32 as an adhesin specific for binding to fibronectin and GAGs via its N-terminal domain (Fischer et al., 2006; Kim et al., 2004; Moriarty et al., 2012; Norman et al., 2008; Probert and Johnson, 1998; Seshu et al., 2006). In Chapter III, we reported that BBK32 recognizes the human complement protein C1r, a modular serine protease, which is essential for the activation of the classical complement pathway. The binding of BBK32 to C1r exhibits a high affinity (K_D ~ 10 - 20 nM) that inhibits its enzymatic activation, blocks the activation of the classical pathway *in vitro*, and impairs classical pathway-dependent killing of *B. burgforferi* (Garcia et al., 2016). Furthermore, we show that the inhibitory effect of BBK32 is mediated by its previously uncharacterized C-terminal domain. To our knowledge, BBK32 is the first bacterial protein that specifically targets complement C1r and subsequently inhibits its enzymatic activity.

It is of interest that a single borrelial lipoprotein, here BBK32 could perform two disparate functions via distinct domains. *B. burgdorferi* has a relatively small

genome and is an obligate parasite that must scavenge many nutrients from the hosts they infect. Hence, *B. burgdorferi* needs to be economical with its limited resources. By producing proteins with multiple functions, *B. burgdorferi* is able to efficiently utilize its energy pools and maintain survival within the hosts it occupies. Since *bbk32* is up-regulated in both feeding ticks and the mammalian environment, the function of this protein as both an adhesin and a complement regulator would seem to simultaneously contribute to the bacterial survival during transmission and colonization. The N-terminal GAG and fibronectin binding domains of BBK32 mediate vascular adhesion and promote borrelial penetration through vascular walls during blood borne dissemination (Moriarty et al., 2012). At the same time, *B. burgdorferi* encounters complement-dependent killing in both feeding ticks where the bacteria confront blood initially, and later in the mammalian circulatory system. Our findings suggest that BBK32 may protect *B. burgdorferi* from the classical pathway-dependent killing by directly binding and neutralizing C1r.

The classical complement pathway is involved in antibody-dependent killing. Thus, the blockage of the classical pathway may not only increase the survival capacity of *B. burgdorferi* in the blood circulating system at early stage of infection, but may also delay antibody mediated neutralization and promote borrelial survival after the humoral immune response develops. *B. burgdorferi* infected mice and humans generate a robust humoral immune response, yet these antibodies are incapable of clearing the infection. In addition, although *B.*

burgdorferi specific antibodies are sufficient to prevent infection and to reduce pathogen burden when passively-transferred to infected antibody-deficient mice (Fikrig et al., 1996; McKisic and Barthold, 2000), protection wanes over time following antibiotic treatment, suggesting that protective adaptive immunity is not long-lived (Elsner et al., 2015; Fikrig et al., 1994; Piesman et al., 1997). It was further shown that *B. burgdorferi* infection disrupts the normal formation of germinal centers (GC), which results in reduced antibody titers, as well as local C4 deposition on follicular dendritic cells (Elsner et al., 2015; Hastey et al., 2012). In this regard, blockage of the classical pathway by BBK32 may be involved in this process by reducing C4 deposition. In addition, disruption of the classical pathway activation may also lead to incompetent complement-dependent antibody killing of *B. burgdorferi*.

Since BBK32 blocks the activation of C1 complement complex *in vitro*, it is important to determine if BBK32 also provides borrelial resistance to complement dependent killing. Wild-type *B. burgdorferi* is intermediately resistant to the complement killing when the spirochetes are incubated with normal human serum (NHS) (Dam et al., 1997; Kochi and Johnson, 1988), possibly due to the presence of BBK32, CspA, and other uncharacterized inhibitors of the complement system (Garcia et al., 2016; Hallström et al., 2013; Kenedy et al., 2009). In contrast, some *B. garinii* strains are sensitive to the killing by NHS; however, depletion of C1q or properdin completely abolished the lethality observed (Dam et al., 1997; Kochi and Johnson, 1988). These data suggest that both activation of the classical and

alternative pathways are involved in complement-mediated killing of serum-sensitive Lyme spirochetes in the absence of *Borrelia* specific antibodies (Dam et al., 1997). As such, resistant isolates, such as *B. burgdorferi,* have developed strategies to evade or inhibit the classical and the alternative pathways to reduce clearance. However, despite the observation that BBK32 potently binds to C1 and C1r and inhibits the classical pathway, we found that the *bbk32* mutant was still able to bind to the C1 complex, and was resistant to complement killing by NHS. This can be explained by the presence of redundant C1 and C1r binding proteins produced on borrelial surface, as well as other unknown complement inhibitors that could compensate for BBK32.

To better characterize the function of BBK32 on *B. burgdorferi* cells, we used a gain-of-function strategy, and cloned full-length BBK32 into the serum-sensitive *B. burgdorferi* strain B314. B314 is missing many borrelial plasmids (including lp36 and lp54 that encode for *bbk32* and *bba33*, respectively) that encode one or several classical complement pathway inhibitors or regulators, which may function with BBK32 together to provide a full-scale resistance to classical pathway-dependent killing. Therefore, the production of BBK32 on B314 cells allows the characterization of BBK32-specific functions. We showed that BBK32 production on the surface of B314 increased borrelial survival of this strain in NHS, but did not provide full-scale protection against complement killing. This can be explained from the standpoint of the pathogen and mammalian host, respectively. First, from the standpoint of the bacterium, the redundancy of *B.*

burgdorferi proteins exists. *B. burgdorferi* produces multiple proteins with similar functions, such as the borrelial proteins binding to C1, and those binding to factor H (Garcia et al., 2016; Kraiczy et al., 2004; Stevenson et al., 2002), to block different stages of complement activation, and ensure an effective inhibition to killing. Therefore, production of a single complement inhibitor such as BBK32 in the B314 background provides incomplete protection. Second, from the perspective of the host, the complement system is also redundant and complicated. Both the classical and alternative pathway are needed for borrelial killing, as the classical pathway recognizes the spirochetes and initiates the activation, while the alternative pathway amplifies the effects of classical pathway. As such, *B. burgdorferi* presumably needs to inhibit both pathways to survive. The lost linear plasmids missing in B314 encode several alternative and terminal pathway regulators, including CspA (Brooks et al., 2005; Hallström et al., 2013; Kenedy et al., 2009; Kraiczy et al., 2000; McDowell et al., 2003a; de Taeye et al., 2013). Therefore, strain B314 fails to inactivate alternative pathway or the final steps of MAC formation. Even when BBK32 is produced in the B314/pCD100 strain to dampen the classical complement pathway activation, the alternative and the terminal pathways are still activated and are lethal to the spirochetes. This could explain why *bbk32* expression in B314 only restored the survival rate to about 40%. To better address the importance of BBK32 in a more physiologically relevant background, we propose to express *bbk32* in a serum-sensitive *B. garinii* strain, which is an infectious Lyme disease spirochete found mainly in Europe.

This strain is sensitive to the classical pathway dependent killing (Dam et al., 1997). The resulting strain could then be tested to determine if the orthologous *bbk32* could partially restore the serum-resistance of this strain to the classical pathway.

Innate immunity is critical for controlling the invasion of pathogenic microbes, since it serves as the first line of host defense. Although the classical pathway was considered as an important adjunct to adaptive immunity since it is best associated with antibody-antigen complex dependent killing, in fact, this pathway is also involved in innate immune defense (Ochsenbein and Zinkernagel, 2000). In addition to the antigen-specific IgG, the C1 complex can be activated by multiple factors including IgM, C-reactive protein (CRP) and serum amyloid P component (SAP), which is the murine counterpart to CRP (Kishore and Reid, 2000). CRP is a major acute phase protein in circulation with dramatically increased levels from less than 10 mg/L in the serum of a healthy human to more than 200 mg/L following the interleukin-6 secretion by macrophages and T cells during acute infections (Ansar and Ghosh, 2013; Thompson et al., 1999). CRP recognizes and bind to lysophosphatidylcholine expressed on the surface of apoptotic cells and some bacteria in order to activate the classical pathway via the C1 complex. Therefore, CRP serves as a potent activator of the classical pathway during bacterial infection.

In addition to CRP, IgM is also a potent activator of the classical pathway. Circulating, naive IgM antibodies (for our purposes, relative to *B. burgdorferi*),

CRP, and SAP all undergo a conformational change when bound to apoptotic cells and foreign structures to reveal a C1q binding site (Agrawal et al., 2001; Chiang and Koshland, 1979). Infection of secreted IgM (sIgM)-deficient mice with different pathogens, including viruses, parasites, and bacteria, has revealed that IgM can mediate innate immune response by either facilitating interactions between pathogens and phagocytic cells, activating the classical complement pathways to lyse the cell, or blocking the pathogen binding to host cells (Boes et al., 1998; Brown et al., 2002; Ehrenstein and Notley, 2010; Hook et al., 2006; Jayasekera et al., 2007; Ochsenbein and Zinkernagel, 2000). After infection, *B. burgdorferi* is able to disseminate to distant tissues through the lymphoid and circulation system, indicating that the spirochetes are able to avoid the lethal effects from these immune responses. The capability of *B. burgdorferi* to resist the serum killing and to survive in NHS is one way to achieve the successful evasion of the innate immune response. The observation that the *B. burgdorferi* strain B314 isolate used in our study, as well as the serum-sensitive *B. garinii*, are susceptible to classical pathway dependent killing in normal human serum that is free of *Borrelia* specific antibody (Dam et al., 1997; Garcia et al., 2016), suggests that the killing is likely mediated by native and immunologically naïve IgM. Previous studies showed that infected ticks that feed on B-cell deficient mice (B6*Igh*$^{-/-}$) harbor more spirochetes than those that feed on wild type mice and passive transfer of either normal mouse serum or natural mouse IgM into B6*Igh*$^{-/-}$ mice reduces spirochete burden in feeding ticks, whereas IgG antibodies have no

effect (Belperron and Bockenstedt, 2001). In addition, we showed that depletion of IgM from NHS restored borrelial survival to similar level of that in EGTA-treated serum (that neutralizes the classical and lectin pathways), suggesting that sIgM and the classical pathway activity are both essential for the killing of serum-sensitive *B. burgdorferi* strain B314. As such, the classical pathway/IgM mediated innate immune response to *B. burgdorferi* may be a key factor to control systemic dissemination and infection following a tick bite. To further understand the role of IgM-mediated classical pathway for controlling *B. burgdorferi* infection *in vivo*, we can determine the infectivity of *B. burgdorferi* in RAG-$1^{-/-}$ mice and $C1q^{-/-}$ mice, respectively. These data may provide additional insight into how *B. burgdorferi* succeeds in disseminating to multiple organs and causes systemic symptoms. Through use of appropriate mutant strains of *B. burgdorferi*, it may be possible to decipher the functions of BBK32 and other putative classical pathway inhibitors involved in this process.

In conclusion, Lyme disease is a significant and re-emerging disease that is a major public health issue in endemic areas. The etiologic agent of Lyme disease, *B. burgdorferi,* produces lipoproteins on the bacterial surface under conditions that simulate the mammalian host, many of which play a role in borrelial pathogenesis. BBA33 and BBK32 are both borrelial surface lipoproteins and contribute to *B. burgdorferi* pathogenesis but in entirely different ways: BBA33 mediates the bacterial colonization and potentially interacts with collagen and collagen-like proteins (C1q), while BBK32 promotes borrelial resistance to the

classical complement cascade and provides an adhesive function to host structures. Since BBA33 is essential for the experimental infection of *B. burgdorferi* in the mouse model, further investigation is warranted to determine how BBA33 functions in borrelial colonization in the skin and dissemination from the skin to distant tissues. BBA33 may also be involved in evasion of the innate immune response based on its ability to bind to host structures that may "cloak" *B. burgdorferi* from the innate and adaptive immne responses. On the other hand, to our knowledge, BBK32 is the first borrelial lipoprotein that specifically inhibits the classical complement pathway via binding to the C1r protein and blocks its enzymatic activity. The ability of the BBK32 C-terminal fragment to inhibit the C1 activation is particularly intriguing given that several pathogenic conditions are (in part) the result of enhanced activation of C1. Assuming that a structure can be resolved for the BBK32::C1r interaction, it may be possible to develop an inhibitor that mirrors this interaction. Taken together, this dissertation has extended our understanding of borrelial lipoproteins in *B. burgdorferi* pathogenesis and, in regard to the BBK32 components, provides a potential tool to target pathologies that involve C1 activation.

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