PROTECTIVE EFFICACY OF A *Borrelia burgdorferi* BB0172 DERIVED-PEPTIDE BASED VACCINE FORMULATION IN THE MURINE MODEL AGAINST LYME DISEASE

A Dissertation

by

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ABSTRACT

Lyme disease (LD) is the most prevalent tick-borne disease in the US. The disease in humans is characterized by the development of a characteristic skin rash (erythema migrans), arthritis, cardiac and neurological signs. Vaccination is the most efficient preventive measure that could be taken to reduce the incidence of the LD around the globe; however at present no vaccine is available for human use. To date, extensive research has been devoted to develop a protective vaccine for use in humans. Nonetheless, it is challenging to find a conserved antigen to combat the heterogeneity within the Lyme spirochetes. Also, the complex biology of *Borrelia* species and alterations in the expression of outer surface membranes, put another burden to generate effective LD vaccines. In this work, PepB, a BB0172-derived peptide was evaluated in scaffolded and conjugated formulations as a vaccine candidate in murine model of LD.

Overall, we observed that, when animals are immunized with pepB conjugated to the tetanus toxoid, they develop high antibody titers that induce protection when animals are infected by needle inoculation. Furthermore, sera from immunized individuals showed bactericidal properties. Finally, the peptide conjugations tested did not protect against the tick infection by clearing infection, but a significant reduction in bacterial burden was observed in immunized groups compared to control groups. Therefore, we conclude that PepB conjugated antigens can serve as an alternative to prevent Lyme disease; nevertheless further studies will be needed to evaluate further delivery methods as well as antigen presentations (polyvalent vaccines, microencapsulation, etc.).

DEDICATION

To my father Salim Hassan, who has always encouraged me to achieve my goals.

To my beloved mother, Turkiya Azzez, who encouraged me and who never stopped believing in my pursuit of this work.

To my loving and supportive wife, Moshriqa Almahmood, and our lovely angels Ahmed, Noor and Sama. Without their love and support of years attending school, this would not have been possible.

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NOMENCLATURE

LD	Lyme disease
CDC	Centers for Disease Control and Prevention
ELISA	Enzyme-Linked Immunosorbent Assay
HRP	Horseradish Peroxidase
Ig	Immunoglobulin
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline with 0.1% Tween-20
DIVA	Differentiating Infected and Vaccinated Animals
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
ID_{50}	50% Infectious Dose
HBSS	Hank's Balanced Salt Solution
BSK-II	Barbour-Stoenner-Kelley II
TTHc	Tetanus Toxoid Heavy Chain
CRM197	Cross Reacting Material 197
TBDs	Ticks and tick-borne diseases

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Lyme disease (LD) is a zoonotic tick-borne disease caused by bacterial spirochetes within the *Borrelia burgdorferi* sensu lato complex. LD was originally recognized in 1976 in Lyme, Connecticut in the United States based upon prevalence of rheumatoid arthritis among children [1]. LD is the most prevalent vector-borne disease in both Europe and North America. In the US, more than 30,000 cases of LD are reported annually to the Centers for Disease Control and Prevention (CDC). On the other hand, according to the European Centre for Disease Prevention and Control (eCDC), around 85,000 cases are reported annually in Europe. LD in humans is characterized by diverse dermatologic, neurologic, rheumatologic, or cardiac abnormalities. The disease is prevalent in temperate regions of the world (North America, Europe, and parts of Asia), where associated competent *Ixodes spp.* tick vectors are present.

The causative agent of LD, *B. burgdorferi* sensu lato complex, is classified within the Phylum Spirochaetes, Order Spirochaetales, Family *Spirochaetaceae*, and includes over 20 different genospecies, of which some have not been reported to cause disease [2]. Nevertheless, Lyme disease in humans is primarily caused by three genospecies of *B. burgdorferi sensu* lato complex, which include *Borrelia burgdorferi, B. afzelii and B. garinii* [3]. These spirochetes are found geographically throughout the temperate regions of the world. In Europe and Asia, *B. afzelii*, *B. burgdorferi sensu stricto*, *B. bavariensis*, and *B. garinii* are the most common genospecies that cause Lyme

borreliosis, whereas *B. burgdorferi s.s.* is the main etiologic agent of LD in North America [4]. However, a novel genospecies *B. mayonii* has been recently described to cause LD infection, and was isolated from patients in the upper midwestern US. *B. mayonii* causes different clinical outcomes as compared to infection with *B. burgdorferi* sensu stricto, such as nausea, vomiting, diffuse macular rash, and high number of circulating spirochetes in the blood, similar to what is observed in relapsing fever Borrelial infection[5]. It has been shown that LD spirochetes have different tissue tropism as *B. burgdorferi s.s.* is mainly arthritogenic, *B. afzelii* causes skin infections, and *B. garinii* is mainly neurotropic [6].

Etiologic organisms, vectors and pathogenesis

Ixodes ticks are responsible for transmitting Lyme *Borrelia* infection. The main tick vectors in Europe is *Ixodes ricinus* [7], whereas in Asia the vector is *Ixodes persulcatus* [8]. In the United States, LD is commonly present in the northeastern and upper midwestern states. In these regions of the country, the disease is transmitted by *Ixodes scapularis* ticks, while *Ixodes pacificus* is responsible for the transmission of *B. burgdorferi* in the Pacific coast states [9]. In addition, the blacklegged tick (*I. scapularis*) is also responsible for transmission of other human pathogens including those causing anaplasmosis, babesiosis, Relapsing fever *Borrelia miyamotoi*, ehrlichiosis, and Powassan virus disease [10]. *Ixodes* ticks are distributed throughout the northern hemisphere [11]. Their life cycle comprised three blood-feeding life stages including larva, nymph, and adult, and it is completed in an average 2-year period. *Ixodes* spp. larvae are naïve after hatching and they acquire the LD spirochetes when

they feed on an infected reservoir host; furthermore, this bacterium is maintained to the subsequent stages, this is also known as trans-stadial transmission [12, 13]. Thus, after molting, infected nymphs are able to infect naïve or infected competent hosts during the next blood meal, spreading the LD causative agent. In fact, ticks in different developmental stages are able to acquire *Borrelia* and transmit Borrelial cells to other susceptible hosts. However, in northeastern US, adult ticks are not significant in disease transmission and maintaining *Borrelia burgdorferi* in its enzootic cycle, since adult females feed primarily on large mammals such as deer, which is important for tick mating [14], but are not competent hosts for the pathogen.

Most ecological studies have been done in northeastern US. Under these environmental conditions, nymphs are generally responsible for the transmission of Borrelial pathogens to humans and companion animals, especially in late spring and summer seasons. *I. scapularis* ticks feed on a wide range of hosts, ranging from small mammals such as mice and rabbits, to larger mammals such as foxes, skunks and white-tailed deer. Humans, dogs and horses are considered incidental hosts, and may develop signs of *B. burgdorferi* infection and disease [15, 16]. Similar to what is observed in other Ixodid ticks [17], the feeding process of the adult female *Ixodes scapularis* tick can last up to 12 days, while nymphal stages can be feeding for up to 8 days. Therefore, in humans, the risk of LD increases after 36 to 48 hours from tick attachment. Conversely, studies have found that in animal models, spirochetal transmission can happen in less than 16 hours post tick attachment [18].

In the tick vector, spirochetes are acquired while feeding from an infected host. Spirochetes will colonize, and subsequently retained in the tick midgut through the different developmental stages (trans-stadial transmission). When infected ticks attach to a naïve host, *B. burgdorferi* will migrate from the midgut to the salivary glands of ticks upon feeding, and from there, to the mammalian host skin. That migration is facilitated by the differentially regulated borrelial outer surface proteins A and C (OspA and OspC) [19-21]. *Borrelia burgdorferi* cells in the tick midgut express high amount of OspA which acts as a docking protein that will interact with the *T*ick *R*eceptor for *OspA* (TROSPA) anchoring *B. burgdorferi* to the tick [19]. Upon feeding, OspA expression is downregulated [19, 22] allowing Borrelial cells to be released from the midgut, migrate to the hemolymph and from there to the salivary glands where they can get injected in the host. At that stage, OspC expression is upregulated, allowing the binding of tick saliva proteins to the surface of borrelial cells, protecting *B. burgdorferi* from the host immune system, aiding in dissemination once in the host [23, 24]. Homologs genes of TROSPA have been identified not only in *Ixodes scapularis* (vector of *B. burgdorferi* in the USA), but also in *I. ricinus*, the tick species transmitting the infection in Europe [25], and *I. persulcatus*, the species transmitting *Borrelia* in Asia [26].

As mentioned above, *B. burgdorferi* has the ability to alter its gene expression as it migrates from the tick vector to the vertebrate host. This differential gene expression is key to ensure their survival in disparate environments found along its life cycle [21, 27]. Once the infected tick starts its blood meal and blood flows inside the midgut, *B. burgdorferi* downregulates the expression of OspA and release spirochetes from

TROSPA. At the same time, there is upregulation of the borrelial OspC, and the bacteria signal cellular division while they migrate to the salivary glands. Overall, a number of genes will be differentially expressed in *B. burgdorferi* as it migrates from the tick to the host in order to adapt to a changing environment. This cascade of events enhances bacterial migration to the tick salivary glands, and then transmission to the mammalian host. After localizing to the salivary glands, *B. burgdorferi* is inoculated with the tick saliva into the host. During feeding, ticks are able to suck up blood to meet their nutritional need, and saliva with excess water and ions that are not essential for the tick are streamed back to the host, generating cycles of sucking and spitting that allow the transmission of pathogens [16, 28]. Examples of differentially expressed genes in *B. burgdorferi* are summarized in Table 1-1.

Tick feeding is facilitated by tick saliva, which contains many components that allow ticks to cement to the host and acquire its blood meal. In addition, tick saliva has a significant role in transmission of spirochetes. Saliva secretions have several compounds that have different functions including reduce pain sensation, reduce inflammation at the bite site, increase blood flow, and anticoagulants that will facilitate pool feeding. In the presence of saliva proteins, ticks feed by damaging host's skin and taking up blood that pools under the host tissues [29-32]. It has been found that tick saliva is able to enhance transmission and infection of *B. burgdorferi* versus studies where the inoculated spirochetes are not associated with saliva proteins [33-35]. For example, the salivary protein Salp15 binds to spirochete via interaction with its outer surface protein C (OspC), which protects the spirochete from antibody-mediated killing, and also inhibits

activation of CD4 (+) T cells [35-37]. Therefore, OspC has been shown to facilitate transmission and infection of *B. burgdorferi* into host organisms [35, 38]. Another example of saliva protein is the tick salivary lectin pathway inhibitor (TSLPI). This protein enhances transmission and acquisition of *B. burgdorferi* by inhibiting the host lectin complement pathway, resulting in reduced chemotactic and phagocytic activity of neutrophils, and diminished spirochete lysis [33].

Table 1-1: Major differentially regulated genes in *Borrelia burgdorferi*

Gene	Location	Host	Function	References
ospA (outer surface protein A)	lp54	Tick	Lipoprotein; binds Tick Receptor for OspA (TROSPA)	[27, 39, 40]
bptA (*Borrelia persistence in ticks A)*	lp25	Tick/ Mammals	Lipoprotein; necessary for tick persistence	[41, 42]
bicA (*Borrelia* iron- and copper- binding protein A) formerly *napA*	Chrom	Tick	Necessary for persistence in ticks	[43, 44]
bba52	lp54	Tick	Outer membrane protein; functions in transmission	[45]
bb0365	Chrom	Tick	Lipoprotein; necessary for persistence in tick	[46]
ospC (outer surface protein C)	cp26	Tick	Immune evasion; required for infectivity	[27, 39, 40]
vlsE (variable major protein-like sequence, expressed)	lp28-1	Mammals	Immune evasion; required for infectivity, expressed after OspC	[39, 47]
dpbA/B (decorin binding protein A/B)	lp54	Mammals	Adhesin; binds decorin/glycosaminoglycans	[48]
ospE (outer surface protein E)	cp32	Tick/ Mammals	Adhesin/Immune evasion; Binds Factor H	[49]
ospF (outer surface protein F)	cp32	Tick/ Mammals	Adhesin; binds heparan sulfate/glycosaminoglycans	[50]
bb0172	Chrom	Tick/ Mammals	Adhesin; binds integrin α3β1	[51]
bbk32	lp56	Tick/ Mammals	Adhesin/immune evasion; fibronectin binding protein	[52, 53]
cspA (complement regulator-acquiring surface protein A)	lp54	Tick	Immune evasion; Binds Factor H	[54, 55]
p66	Chrom	Tick/ Mammals	Adhesin/Porin; Binds β3 chain integrins	[56, 57]
pncA (pyrazinamidase/ nicotinamidase)	lp25	Tick/ Mammals	Metabolism; Nicotinamidase activity	[58, 59]
bba34 (oligopeptide permease A5)	lp25	Tick/ Mammals	Transporter; Putatively binds Sodium Acetate or Sodium Bicarbonate	[60]

Chrom = Chromosomally encoded; lp= linear plasmid; cp= circular plasmid

Tick histamine release factor (tHRF) is another component of tick saliva that stimulates histamine release in the host, leading to increased blood flow and vascular permeability. The tHRF has been shown to have a role in tick feeding and borrelial transmission in mice [61]. Therefore, it is important to consider a method of borrelial infection in vaccine studies, as there is a significant difference when animals are challenged with needle protocol vs using tick model of infection [62].

Differential gene expression of Lyme disease spirochetes

The changes in gene expression of spirochetes is the key role for their survival in two disparate environments, the tick vector and vertebrate host. In unfed tick conditions, spirochetes reside in the tick midgut, and during feeding the flowing of blood in the midgut induces migration of spirochetes to the salivary glands, and then transmission to mammalian host. At the same time, through differential gene expression, spirochetes successfully adapt to the new mammalian host environment. In laboratory setting, to study differential gene expression of spirochetes requires conditions similar to those described above. To mimic flat tick conditions, spirochetes are grown in BSK-II media at 23°C and a pH of 7.6. On the other hand, to mimic tick feeding conditions, bacteria are incubated at 37°C and a pH of 6.8. To study the pattern of gene expression, spirochetes grown and adapted to flat-tick conditions are shifted to grow under feed tick conditions, and the spirochetes can be analyzed before adaptation [27, 39, 42, 44, 55, 63, 64]. Therefore, this is an *in vitro* strategy that can help understanding differential gene expression without the need to use ticks.

The outer membrane of *B. burgdorferi* lacks the lipopolysaccharide found in gram negative organisms, instead *B. burgdorferi* has a high density of lipoproteins, some which have been shown to be differentially expressed [42, 65, 66]. These outer surface proteins are critical for bacterial survival, metabolism, and adhesion within the vector and/or host environment [67]. Also, surface exposed outer proteins are immunogenic and play a vital role in bacterial pathogenesis; therefore, they are effectively considered as targets for anti-Lyme vaccines [68].

Historically, the development of LD vaccine has been based on modifications of either the outer surface protein A (OspA) or C (OspC). The first and only licensed human Lyme vaccine was an OspA based vaccine, LYMErix by SmithKline Beecham [69]. It was withdrawn from the market in 2002 due to lack of interest and potential side effects associated with its use. This vaccine formulation targeted the Borrelial OspA lipoprotein, which is expressed when the pathogen is in the arthropod tick vector. When expressed, OspA mediates the attachment of spirochetes to the tick mid-gut via its receptor (TROSPA) [19]. The other important differentially expressed protein is outer surface protein C (OspC). This lipoprotein is expressed during mammalian infection, and it facilitates transmission of *Borrelia sp.* from the tick vector to the mammalian hosts [70]. The OspC has been used in several anti-Lyme vaccine formulations in dogs [71-76]. Other examples of products of differentially regulated genes of *B. burgdorferi* that have been proposed as vaccine candidates are decorin binding protein A (DbpA), BBK32, and BB0172 [77-79]. DbpA and BBK32 are upregulated proteins within the mammalian host, and are members of the microbial surface components recognizing

adhesive matrix molecules (MSCRAMM) protein family, and they mediate binding of *B. burgdorferi* to extracellular matrix proteins of the host. Therefore, they enhance bacterial virulence and pathogenesis through colonization and dissemination of spirochetes in the host [80, 81].

B. burgdorferi has the variable major protein-like sequence, expressed (VlsE), which is a surface protein characterized by antigenic variation. This is an immunodominant antigen expressed by *B. burgdorferi* when growing in mammalian tissues. In brief, the *vls* (Vmp- like sequence) locus is located on a 28-kilobase linear plasmid (lp28-1) of *B. burgdorferi* B31, and comprises an expression site (VlsE) and 15 non-coding *vls* cassettes. Different antigenic variants of VlsE are expressed by recombination between segments of the expression site and the silent cassettes [82, 83]. Therefore, VlsE play a role in evasion of the host immune system and persistence in the infected host. It has been predicted that over 120 distinct potential lipoproteins are encoded by about 8% of *B. burgdorferi* B31 genome [84].

Pathogenesis of Lyme disease

Lyme disease (LD) is a multi-systemic disease caused by infection with the spirochetal bacteria *Borrelia burgdorferi* sensu lato (sl), and it is characterized by fever, arthralgias, myalgias, and erythema migrans [85]. In humans, the early infection with *Borrelia burgdorferi* sl, also known as localized infection, is characterized by appearance of an erythematous skin lesion known as Erythema Migrans (EM, the bulls-eye rash) at the site of the tick bite [86]. The EM lesion appears within 1-30 days after tick bite in about 75% of the patients [6]. If the disease is not treated, it progresses to the

second stage of the early LD, or disseminated infection. In this state, the disease is characterized by systemic symptoms of infection such as fever, malaise, headache, myalgias, arthralgias, and dizziness [87, 88], also known as flu-like symptoms. In this state, cardiac involvement may be present, and some infected individuals show neurological signs within weeks to months following infection. The clinical picture of the patients in the third stage of LD, or persistent infection, is mainly manifested by arthritis. This stage usually develops following several months to years after the initial infection with Lyme *Borrelia* species [11, 89]. In fact, Lyme arthritis in children was the cause for the recognition and identifying of LD by Dr. Alan Steere in Connecticut in 1977 [1]. It is estimated that about 60% of untreated patients develop Lyme arthritis manifested clinically by joint pain and swelling, and it is especially affected large joints such as knee [90].

Pathogenesis of Lyme arthritis is initiated when spirochetes are frequently localized in joints or periarticular tissues during the dissemination phase of infection [91]. In most cases, the early stages of Lyme arthritis are asymptomatic, but later due to stimulation of antibody production and inflammatory cells response, the signs of clinical arthritis become evident [92]. The infected individuals respond to antibiotic treatment especially during the early stages of infection; however, if the treatment of LD is neglected, it will lead to sever rheumatologic, cardiac, and neurologic complications. Recently, and after many years of controversy [93], Post-Treatment Lyme Disease Syndrome (PTLDS) has been characterized by researchers at John Hopkins [94]. In this study, authors conclude, that even though physical exams and laboratory tests show little

abnormalities in PTLDS patients, standardized symptom questionnaires, showed significantly poor health related quality of life compared to control groups. In addition, PTLDS patients presented with musculoskeletal pain, sleep disorder, depression and fatigue that could be detected only when using the so mentioned standardized questionnaires. Thus, PTLDS is a chronic illness that occurs following standard antibiotic treatment for early or late LD, and it has been reported in about 10-20% of treated patients [95].

Diagnostic measures of Lyme disease

In LD, the infection can be subclinical without any symptoms, but in clinical disease cases, signs such as EM, arthritis, neurological signs are apparent for clinical diagnosis. Microbiological isolation is still the 'gold standard' for diagnosis of bacterial infections. However, some infectious agents such as *Borrelia burgdorferi* cannot be isolated on selected media in a timely manner and compatible with diagnostic efforts. Therefore, alternative diagnostic methods can be used such as direct detection of pathogen, indirect serological tests, and other molecular techniques such as PCR [96, 97]. Lyme borreliosis is a disease with diverse clinical manifestations, and it can be misdiagnosed especially if physicians are not aware of the full picture of the LD. The CDC recommends using a two-tiered serological protocol as the most reliable diagnostic approach to increase test specificity in the lab diagnosis of LD samples. In the two-tiered protocol, the serological Enzyme Immuno Assay (EIA) is used to screen sera from infected individuals. EIA such as ELISA, chemiluminescence immunoassays (CLIA), and electrochemiluminescence immunoassay (ECLIA) are used due to their versatile

ability to detect specific IgG and IgM antibodies [98]. Initially, the first step of the protocol includes utilizing an enzyme immunoassay (EIA) to detect antibody response against either the VlsE C6 antigen or whole-cell *Borrelia* lysate. Then, confirmation of the positive or borderline results with the second tier, consisting in immunoblotting against whole-cell *Borrelia* lysate or other significant antigens [99-101]. In Europe, the validity of using a single serological test in diagnosis of Lyme borreliosis is questionable regarding its adequate sensitivity and specificity to use it alone [102-105]. The design of a single immunoassay to detect Lyme borreliosis is challenging due to presence of high biodiversity of *Borrelia* genospecies in Europe [103, 105]. Therefore, other serological tests such as indirect immunofluorescence, complement fixation and indirect hemagglutination assays are currently deemed inappropriate to use for diagnosis of LD [100, 106].

In general, duration of the disease will determine the dominant immunoglobulin isotype at the time of testing; therefore, serum will be evaluated for either IgM or IgG antibodies based on whether the individual is early or latter in the infectious process. Basically, immunoblotting is used to test samples for IgM in the early stage of disease, less than or equal to 30 days. Whereas, IgG antibody can be detected later in serum samples [107]. Immunoblot results for IgM are considered positive if a sample shows at least 2 of 3 signature bands (OspC, BmpA, Fla), and 5 of 10 signature bands for positive IgG results (OspC, BmpA, Fla, and antigens designated by their molecular weights: 18, 28, 30, 45, 58, 66, and 93 kDa proteins) [108-110].

Current methods for the serological diagnosis of LD have few limitations such as lag of seroconversion by weeks following infection, inability to recognize active infection, past infection or reinfection, and no predictive value to track progression of acute LD to a chronic state [111]. Therefore, researchers try to develop new diagnostic methods that can overcome these restrictions. Among these tools, detection of DNA by molecular approach can be used for diagnosis of LD such as multiplex real-time PCR [112], and next generation sequencing (NGS). The multiplex assays allow for detection of multiple pathogens simultaneously by using a variety of probe fluorophores that can be detected at different wavelengths. Multiplex real-time PCR assay is useful to screen samples for more than one tick-borne disease pathogen at a time [113]. In a study involved human blood samples, multiplex qPCR was used to detect *Babesia microti* and *B. burgdorferi*. The results of that study showed 80% sensitivity for detection of LD and *B. microti* co-infection [114]. In regard to NGS, the various NGS platforms were developed for analyzing full genome sequencing, and identification of potential genetic diseases [115]. Moreover, NGS has been further expanded to include diagnostic applications that can be used to identify various infectious agents[116]. It has been studied that deep sequencing of the hypervariable outer surface protein C locus (*ospC*) was used to detect *B. burgdorferi* in *Ixodes scapularis* ticks collected from New York State, USA. It has been shown from that study that targeting the conserved region of intergenic sequences has significant implications in diagnosis and quantification of LD spirochetes in clinical and wildlife specimens [117]. Furthermore, other indirect methods such as evaluation of some released chemokines during infection and other host protein

biomarkers that support early LD diagnosis. For example, a European study showed that analysis of cytokine CXCL13 in cerebrospinal fluid (CSF) from suspected cases with Lyme neuroborreliosis (LNB) was a useful diagnostic tool. In fact, it supported the LNB diagnosis in children with short symptom duration [118]. Also, ELISpot assay was used for diagnosis of Lyme borreliosis in a clinical study included *Borrelia* positive patients. It showed promising results as a new diagnostic tool with higher specificity and sensitivity compared to the western blot assay [119].

Treatment of Lyme disease

The early manifestations of LD, both localized and disseminated forms, are characterized by spontaneous healing without medical intervention. Treatment in such cases is recommended to avoid the sequelae of the disease, especially neuroborreliosis and Lyme arthritis. In the US, more than 80% of untreated patients developed Lyme arthritis [120]. Whereas in Europe, patients may develop Acrodermatitis Chronica Atrophicans (ACA) [121, 122]. *In vitro* studies have shown that *Borrelia burgdorferi* s.l. strains are susceptible to most penicillin, tetracyclines, many second and third generation cephalosporins and macrolides [99, 123, 124]. Early localized LD can be effectively treated with doxycycline, phenoxymethylpenicillin and amoxicillin, whereas the early disseminated form is treated with intravenous injection of ceftriaxone or penicillin. However, researchers have found that an oral administration of doxycycline is effective for treatment of Lyme neuroborreliosis in Europe [125, 126]. However, applying prophylactic measures is the primary method of preventing LD infection, such as avoid

tick-populated areas, wearing long sleeved and long pants clothing, tick observation, and use of repellents [127, 128].

Prevention of Lyme disease

Ticks are ranked second to mosquitoes worldwide for transmitting infectious disease agents to humans [129]. They are transmitting a wide diversity of viral, bacterial, and protozoal infections among animals and humans. In many temperate regions around the world, tick-borne diseases are prevalent and considered the most important vector-borne infections. Among these, Lyme borreliosis caused by *Borrelia burgdorferi* and transmitted by *Ixodes spp* ticks. In endemic areas, exposure of susceptible hosts to infected ticks is associated with increased risk of LD infection. Therefore, minimizing the risk of tick bites can significantly reduce the risk of tick-borne diseases. This can be accomplished by applying prophylactic measures such as avoiding tick-infested areas, wearing protective, covered clothing, and applying tick repellents and acaricides. In addition, permethrin treated clothes can be used especially for individuals who stay long period of times in tick infested areas. These clothes showed reduction in tick bites of 93% in people wearing permethrin treated clothes compared with controls [130]. Also, removal of the attached ticks with forceps, especially following hiking, camping, and other outdoor activities [131]. Moreover, school campuses, neighborhood backyards, parks, and other places are considered also important avenues for transmission of tick-borne diseases [132]. The important considerations to prevent tick bites should be educating people about risks associated with tick infestation. One recent study showed

that *Borrelia* infections were reduced in people who were exposed to prior educational opportunities regarding ticks and how to prevent tick bites [133].

Companion animals especially dogs can carry ticks to humans, particularly in endemic areas, as they are considered significant risk factors for disease acquisition because they are more frequently exposed to infected ticks [134, 135]. Therefore, treatment of these animals with anti-tick preparations and or available canine Lyme disease vaccines are recommended [128].

Lyme disease Vaccine Development

Immunization studies started shortly after discovery of *B. burgdorferi* in 1977. Johnson and collaborators showed that passive transfer of immune rabbit serum to hamsters protected against *B. burgdorferi* infection [136]. Later, the same team evaluated immunization of hamsters with whole, inactivated *B. burgdorferi*. In brief, naïve hamsters were immunized with a single dose of inactivated *B. burgdorferi* and challenged with a dose of 1,000 × ID_{50} *B. burgdorferi* cells. The group of animals which immunized with 50 µg of *B. burgdorferi* cells (dry weight) showed 86-100% protection [137]. Therefore, these studies have shown that humoral immune response is responsible for protection against LD. In addition, the study of Schmitz and collaborators [138] showed that passively immunized hamsters with serum from infected animals were protected from development of arthritis. Moreover, serum ability to confer protection was associated with the development of borreliacidal antibodies [138, 139]. Collectively, those studies showed that humoral immune response induced by vaccination is able to confer protection against infection with *B. burgdorferi*.

Since then, researchers started focusing their efforts on identifying major epitopes of *B. burgdorferi* that are able to induce a protective immune response. Of these, outer surface proteins gained significant interest. Previous studies revealed that immunized mice with OspA, either by active or passive routes, were protected from infection with *B. burgdorferi* [140, 141]. In addition, it has been shown that removal of OspA antibodies from Lyme arthritis patient's serum, was unable to confer protection in passively immunized hamsters [142]. Likewise, it has been shown that severe combined immunodeficiency mice (scid) receiving OspA-specific monoclonal antibody were protected against *B. burgdorferi* infection [143]. Therefore, the above studies provided support to use OspA-based vaccines in human and companion animals. Historically, the development of LD vaccine has been based on modifications of either the outer surface protein A (OspA) or C (OspC). The first and only licensed human Lyme vaccine was an OspA based vaccine, LYMErix by SmithKline Beecham [69]. It was withdrawn from the market in 2002 due to lack of interest and potential side effects associated with its use. This vaccine formulation targets the Borrelial OspA lipoprotein, which is expressed when the pathogen is in the arthropod tick vector.

During tick feeding *B. burgdorferi* migrated to the salivary glands of ticks, and then to the mammalian host. That migration is facilitated by the downregulation of OspA expression upon blood intake [22, 144]. Thus, OspA based vaccines are effective in conferring protective immunity by blocking the transmission of *B. burgdorferi* from the tick to the mammalian host [145]. In fact, the antibodies induced by the OspA vaccine neutralizes *B. burgdorferi* in the tick vector rather than in mammalian host. Furthermore,

administration of OspA formulated vaccine to small rodents (reservoir hosts) has been used to reduce the prevalence of *B. burgdorferi* in the mammalian reservoirs and the tick vectors, and break the enzootic cycle of the disease in nature, which might reduce the incidence of *B. burgdorferi* infection in human [146, 147]. In addition, in veterinary medicine, multiple OspA based vaccines have been used to immunize dogs against LD, such as Nobivac® Lyme from Merk Animal Health; LymeVax® from Fort Dodge and Recombitek® Lyme from Merial [75, 148]. Nevertheless, since specific antibodies have to neutralize *B. burgdorferi* while in the tick vector, individuals immunized with OspA vaccines need to maintain high antibody titers in order to have an effective vaccine. Researches from Valneva, Austria GmbH designed multivalent OspA-based vaccine derived from multiple OspA serotypes, which include *B. burgdorferi* (serotype 1), *B. afzelii* (serotype 2), *B. garinii* (serotypes, 3, 5 and 6) and *B. bavariensis* (serotype 4). Scientists incorporated C-terminal halves from 6 of the most clinically relevant OspA proteins in their vaccine candidate, but without including the hLFA-1 epitope [149]. This vaccine formulation, referred to as VLA15, has been tested in Europe through Phase I/II clinical trials, and it was showed to be safe and immunogenic [150, 151]. In addition, VLA15 conferred protection to immunized mice against challenge with four different *Borrelia* species [152]. Therefore, Phase III clinical trials will be pursued with the intention of evaluating efficacy [150].

The other important differentially expressed protein during the transmission of *B. burgdorferi* from the tick vector to the mammalian host is outer surface protein C (OspC). This lipoprotein is expressed during early mammalian infection (upon blood

meal in the tick midgut until approximately 10 days post-transmission), and it facilitates transmission of *Borrelia sp.* from the tick vector to the mammalian hosts [70]. The OspC shows significant heterogeneity within *Borrelia sp.* isolates [153]. Therefore, in order to use a protective vaccine, any construct of OspC based vaccine is required to incorporate the heterologous potentially protective epitopes from different *Borrelia sp.* isolates. In fact, to generate a complete, broadly protective vaccine against LD using the OspC antigen, 34 distinct epitopes would be required [154]. Based on such observations, an octavalent**,** and chimeric OspC vaccine candidate has been evaluated [155], and a recombinant, heptavalent LD vaccine has been developed by Zoetis, VANGUARD ® crLyme, formulated to prevent LD in dogs [156]. It has been shown that OspC is an essential factor for establishing mammalian infection [157]. Hence, it induces immune response upon infection. Several studies showed that sera from patients with early LD have significant extensive reaction to OspC compared to OspA [110, 158, 159]. Moreover, it has been shown that patients with erythema migrans have prominent IgM response to OspC [110]. In addition, patients with various early LD manifestations showed moderate IgG response to OspC [160]. These findings reinforce the continued use of OspC as a vaccine candidate.

LYMErix by SmithKline Beecham

The U.S. Food and Drug Administration (FDA) approved the first recombinant OspA vaccine against LD, LYMErix by SmithKline Beecham (now GlaxoSmithKline) in December 1998. The vaccine was derived from the lipoprotein outer surface protein A (OspA) of *B. burgdorferi*. It was withdrawn from the market in 2002 due to lack of

interest and reported side effects [161]. Another recombinant OspA vaccine, ImmuLyme was generated by Pasteur Mérieux-Connaught (Swiftwater, Pennsylvania), and it was proved to be safe during phase I and phase II studies. However, ImmuLyme was not introduced to the public due to the company not proceeding to apply for licensure [162, 163]. The LYMErix vaccine was designed for individuals aged 15-70, while ImmuLyme for individuals aged 18-92 [164]. Characteristics of human LD vaccines (LYMErix™ and ImuLyme™) are listed in Table 1-2.

The LYMErix vaccine was instructed to use with aluminum hydroxide as an adjuvant, in 3 doses, and showed protection in about 80% of vaccinated individual against infection with *B. burgdorferi* [165]. The main drawback of this vaccine was to maintain high titer of OspA antibodies in the host, required to confer protection against borrelial infection [145, 166-168], since the antibodies will block the transmission of *B. burgdorferi* while the pathogen is inside the infected ticks.

Table 1-2: Human LD vaccines at Phase III clinical trial evaluation

Vaccine	Company	FDA License	*Borrelia* Antigen	Adjuvant	Efficacy	Route of administration	References
LYMErix™	SmithKline Beecham	Yes	Full length, lipidated OspA strain Z S7	Lipidation,Aluminum Hydroxide	76%	IM	[169, 170]
ImuLyme™	Pasteur Mérieux Connaught	No	Full length, lipidated OspA strain B31	Lipidation only without adjuvant	92%	IM	[163, 171]

Both vaccines were administered intramuscularly at a dose of 30 μg and a frequency of 0, 1, and 12 months with required annual boosts.

It has been suggested that structural similarity between some host proteins and OspA can induce an autoimmune reaction, which resembles Lyme arthritis [172]. It has been revealed that part of OspA (amino acids: 165-173), the immunodominant T cell epitope, has similarity to the human leukocyte function associated antigen-1 (hLFA-1) (amino acids: 332-340) [173, 174]. The *HLA-DRB1*0401* and *HLA-DRB1*0404* are found to present the $OspA_{165\text{-}173}$, and its hLFA-1 homolog to T-helper cells, and induce an autoimmune reaction characterized by antibiotic resistant Lyme arthritis [174-177]. Prior to approval of the OspA vaccine field trials by the FDA, it was known, that vaccination with *B. burgdorferi* or its components may induce potential side effects such as arthritis. For instance, previous studies showed that immunized hamsters with OspA based antigen, developed arthritis following infection with *B. burgdorferi* [178]. In addition, OspA vaccination was correlated with cases of arthritis and neurological signs in humans [179, 180]. Furthermore, several reports have shown that vaccination with

whole *Borrelia* organisms are associated with severe arthritis following challenge of immunized mice with *B. burgdorferi* [181-184].

Lyme disease vaccines for domestic animals

Lyme borreliosis can also infect dogs and horses beside human. Infected ticks are able to transmit the infection to dogs and other animals as well. Companion animals primarily dogs are under the risk for tick exposure due to their outdoor activities. In dogs, the clinical manifestations are characterized primarily by Lyme arthritis, lameness, and some may develop Lyme nephropathy, although the majority of seropositive cases of Lyme borreliosis are asymptomatic or subclincally infected [185]. However, most cases of equine infection are asymptomatic [186, 187]. Nevertheless, the clinical disease in horses is characterized by neuroborreliosis, uveitis, lethargy, lameness, hyperesthesia and cutaneous pseudolymphoma [186, 188, 189]. In the US, seroprevalence studies of *B. burgdorferi* antibodies circulating among adult horses in endemic areas showed relatively high rates of 33% or greater indicating previous exposure to Lyme spirochetes [187, 190]. Also, Lyme borreliosis in cats is usually characterized by inapparent infection; however, the clinical signs may include fever, lethargy, and lameness [191-193]. Whereas in cattle, infections with *Borrelia*-like spirochetes are rare, and can cause laminitis, stiffness and swollen joints, reduced milk yield, weight loss, and abortion in pregnant animals [194, 195].

Currently, vaccine against Lyme borreliosis is available only for dogs, and there are 5 commercial licensed vaccines to use in North America, produced by 4 different companies [196]. A list of vaccines used for dogs can be found in Table 1-3.

Table 1-3: Available commercial vaccines for dogs against Lyme disease [196]

Name of vaccine	Company	*Borrelia* Antigen	Adjuvant
Recombitek® Lyme	Merial	Recombinant OspA(monovalent)	OspA Lipidation
LymeVax®	Zoetis	Bivalent whole –cell inactivated Bacterin (OspA containing strain and OspC strain)	Aluminum hydroxide
Duramune® Lyme	Elanco	Bacterin (OspA containing strain and OspC strain)	Proprietary
Nobivac® Lyme	Merck	Bacterin (OspA containing strain and OspC strain)	Oil
Vanguard® crLyme	Zoetis	Chimeric recombinant (monovalent OspA and 7 types of OspC)	Aluminum hydroxide

Wildlife vaccination Trials

Zoonotic pathogens such as Lyme disease circulate between human and animal hosts including wildlife reservoir hosts. Therefore, preventing the transmission of pathogens in the vertebrate reservoir and/or within the arthropod vector is one of the methods that can be used to reduce the prevalence of vector borne diseases in humans and companion animals. Vaccination of vertebrate reservoir hosts can disrupt the zoonotic transmission cycle of pathogens, and reduce the infection rate in the arthropod vector. In the wildlife habitat setting, oral vaccination is recommended because it is not invasive, and is practical and cost-effective [197]. A bait vaccination approach has been successfully used to control rabies and plague in wildlife animals [198-201]. *B. burgdorferi* circulates in nature between tick vectors of the genus *Ixodes* and vertebrate reservoir species such as white footed mouse, eastern chipmunk, and white-tailed deer

[202-207]. Fikrig and collaborators [208] showed that mice immunized with recombinant outer surface protein A (rOspA) administered orally were protected against *B. burgdorferi* challenge. Another study [197] concluded that oral administration of purified OspA provided protection against systemic infection with *B. burgdorferi* to immunized mice. From this view, immunization of wild white-footed mice *Peromyscus leucopus* with OspA via subcutaneous inoculation reduced in the test sites (Connecticut), both prevalence of *B. burgdorferi* in *I. scapularis* nymphs, as well as human risk of contracting Lyme disease [146]. Such studies provided the baseline to develop an oral bait system to immunize mice with OspA [209] , to reduce pathogen burden in the arthropod vector. In addition, a study by Voordouw et al. [210] showed that oral bait vaccine derived from OspA significantly reduced transmission of *B. burgdorferi* from previously infected white footed-mice to feeding ticks. In addition, in the same mentioned study, the oral bait vaccine conferred protection in naïve white footed-mice against *B. burgdorferi* challenge.

In general, oral vaccination of wildlife is considered a promising tool to reduce prevalence of infectious diseases in both human and animal hosts. However, it requires logistic support on a large-scale basis, from providing large quantities of vaccine, information related to ecogeographic patterns of targeted regions, and vehicles for distribution of baits, personnel and strategic planning. In regard to LD spirochetes, there are several vertebrate reservoir hosts that may not be targeted by the vaccine bait, and even not all the targeted animals will take the bait. The implementation of bait-based vaccines in wildlife habitats might help in the reduction of disease prevalence in those

animals. Therefore, there will be a potential decrease in the numbers of infected tick populations that would reduce the incidence of LD in human and other susceptible animals, however the risk for exposure to LD spirochetes is still high especially in endemic areas. Thus, human LD vaccine is still required to reduce disease prevalence in the endemic and hyper-endemic areas.

Conjugated vaccine

Vaccination is the most effective method used for control infectious diseases. In more recent years, conjugated vaccines have emerged to improve immunogenicity of vaccines to combat a variety of infectious agents. Therefore, a number of molecules have been developed and used as carrier molecules for the generation of protective vaccines against different infectious diseases in humans. Among those that have been evaluated in preclinical and clinical studies, five carrier molecules have been used successfully in licensed vaccines, which includes diphtheria and tetanus toxoids, the Cross Reacting Material (CRM197), the outer membrane complex of *Neisseria meningitidis* serogroup B (OMPC), and the protein D derived from *Haemophilus influenzae* (PD) [211]. CRM197 was isolated from *Corynebacterium diphtheria* C7 $(\beta197)^{tox-}$, and it can also be produced by recombinant DNA methods in heterologous organisms. We initially selected tetanus toxoid and CRM197 in our current study because they are commercially available and have been shown to be safe for use in humans [212]. The incorporation of carrier proteins to polysaccharide antigens induces T-cell dependent antibody responses, which enhances immunological memory, longer lasting protection, and enable boosting

of antibody titers with further doses [213]. In addition, not only the strength but also the type of T-helper cell response affects the quality of the T-cell help [214].

OMPC has been used in *Haemophilus influenzae* type b conjugate [215] and pneumococcal conjugate vaccines [216]. PD is a 40 KDa cell surface protein derived from non-typeable *H. influenzae*, and has been used in a multivalent pneumococcal conjugate vaccine [217, 218]. Tetanus toxoid is a detoxified product of tetanus toxin, which is produced by *Clostridium tetani* cultures.

The Cross Reacting Material (CRM197), a non-toxic mutant of Diphtheria toxin with a single nucleotide substitution. In particular, nucleotide 197 was modified from G → A inducing a change from Glycine in amino acid 52 in the wildtype molecule to Glutamic acid in CRM197 [219, 220]. This substitution resulted in loss of function, and therefore makes this molecule safe to be used as an immune system stimulation, and an excellent carrier molecule. Consequently, CRM197 is widely used as a carrier molecule for licensed conjugate vaccines such as in *Haemophilus influenzae* type b (Hib) and *Streptococcus pneumoniae* FDA approved vaccine Prevnar®13 [221]. Conjugate polysaccharide vaccines containing CRM197 as a carrier molecule have been used successfully worldwide to immunize hundreds of millions of people. Also, CRM197 based conjugate vaccines such as Menveo® Menjugate®, and Meningitec® have been approved to use against *Neisseria meningitidis* infection [222]. In addition, several CRM197-conjugate polysaccharide vaccines are under further clinical and preclinical evaluation such as vaccines against *Salmonella enterica* serovar Typhi, *Salmonella*

paratyphi, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Streptococcus agalactiae,* and several pneumococcal conjugate vaccines [223-228].

Contrary to Diphtheria toxin, CRM197 is a 58 kDa protein that is genetically modified to be non-toxic, and therefore detoxification process prior to use is not required. Furthermore, Diphtheria toxoid is immunogenic, but studies have suggested that the inactivation process might affect some T-helper cell epitopes. Thus, since CRM197 does not need to be detoxified through chemical treatment, it has enhanced carrier effect due to its intact T and B cell epitopes [227, 229]. The isolation of different cross-reacting forms of diphtheria toxin (CRMs) began in 1971, and the mutant form CRM197 was isolated in 1973 [230, 231]. The final optimized product was commercially available in the mid-1980s. Characteristics of CRM197 molecule is summarized in Table 1-4.

Table 1-4: Properties of CRM197 molecule

Characteristics	Advantages	References
Widely used in commercially licensed vaccines Large-scale production and non-toxic mutant form of diphtheria toxin Indirect conjugation via a linker molecule Has conformational changes when reacting to monoclonal antibodies Binds to phospholipid bilayers	Binds to different molecules and membranes independent of temperature and pH No need for chemical detoxification Maintain its three-dimensional structure during processing	[229, 232-238]

Anti-tick vaccines

Ticks and tick-borne diseases (TBDs) are distributed worldwide and considered major threats to human and animal health. Ticks can carry wide array of etiological agents including protozoal, bacterial, and viral pathogens. Currently, the CDC has listed 16 tick borne diseases that can infect humans in the US. The most common TBDs in humans include Lyme disease, babesiosis, ehrlichiosis, Rocky Mountain spotted fever, anaplasmosis, tick-borne relapsing fever, and tularemia [239, 240]. In general, zoonotic diseases are of significant importance as they consider a major threat to public health. Specifically, LD is the most prevalent zoonotic tick-borne infection reported in the US. In fact, ticks are considered the exclusive, vital vehicle for transmission of LD to mammalian hosts [241]. Hence, control the expansion of ticks can significantly reduce prevalence of TBDs among humans and animal populations. Based on that, the use of

acaricides to control ticks and the pathogens they transmit has been extensively used, and has shown some limiting factors such as development of tick resistance to these chemicals, and release of environmental contaminants [242-244]. Moreover, there are currently no available effective vaccines against most TBD agents, therefore there is a need to develop new strategies to control ticks and their associated agents [245]. One of these strategies is to interfere with the ticks feeding on mammalian hosts by using putative anti-tick vaccines [246]. In the absence of effective anti-tick vaccines, controlling ticks by chemical acaricides is the only reliable method used to eliminate these ectoparasites and protect animals, while humans are left with insect repellents, protective clothing and tick checks after outdoor activities as their only protection against TBDs [247, 248]. Several studies have evaluated the feasibility of immunizing animals with tick antigens, and study the effect of tick immunity on pathogen transmission and acquisition. Of special interest have been saliva proteins, due to their role in tick feeding and immune system evasion. Some researchers have evaluated the potential role of Salp15, a 15 kDa secreted salivary protein from *Ixodes scapularis*, as a potential tick vaccine. Salp15 protein has immunosuppressive traits, as it inhibits activation of $CD4^+$ T cells, complement, and dendritic cells [249]. Thus, in previous studies, anti-Salp15 immunity induced 60% protection against *Borrelia burgdorferi* infection [250] in the mouse model.

Another study showed that immunized mice with Salp25D, a 25 kDa secreted salivary protein from *I. scapularis,* had reduced spirochete acquisition by ticks. This protein has an antioxidant activity, and has been shown to protect *B. burgdorferi* from

the effects of reactive oxygen species produced by neutrophils [251]. Immunized mice with tick histamine release factor (tHRF), secreted salivary protein from *I. scapularis,* have markedly reduced bacterial burden in mice, and efficiency of tick feeding [61]. Moreover, implementing vaccine candidates derived from tick gut antigens had shown effectively reduction of tick engorgement on cattle [252]. On those lines, the study conducted by Kotsyfakis et al. [253] showed that immunized guinea pigs, with sialostatin L2, have reduced the feeding ability of *I. scapularis* nymphs. Moreover, it has been reported that immunized mice with the tick salivary lectin pathway inhibitor (TSLPI) from *I. scapularis* showed impaired bacterial acquisition and persistence in tick midguts, and 30% reduction of bacterial burden in the host [33].

A novel approach: The VWFA domain containing proteins

The Von Willebrand factor A (VWA) domain is well known for its presence in extracellular matrix proteins, integrin receptors, and cell adhesion proteins. Proteins containing these domains are found across Eukarya, Archaea, and Bacteria. Although it is primarily found within extracellular proteins, some proteins involved in essential cell functions, such as DNA repair or transcription, also contain VWA domains. Multiprotein complexes, in particular, commonly feature these domains, and VWA domain interactions may be essential to the assembly or functionality of these complexes. VWA domains exhibit classic Rossmann folds: β-sheets flanked by multiple α-helices in alternating sequence. Many have loops with a noncontiguous sequence motif called a metal ion-dependent adhesion site (MIDAS) where metal ions are bound. Protein-protein interactions at these sites often involve divalent cations such as Ca^{2+}, Mg^{2+} and Mn^{2+}.

VWA domains take part in many important functions, including linkage of platelets to collagen during blood coagulation and interaction of integrins with collagen and other ligands. As such, mutations in these domains can lead to a variety of human diseases [254-256].

Subramanian et al. [257] identified new protein family VWFA domains in both spirochetes *B. burgdorferi* and *Treponema pallidum*. In fact, these novel proteins containing domains only previously recognized in eukaryotes. However, the comparative genome analysis revealed that the *Borrelia* VWFA domain containing proteins were not highly similar to the VWFA proteins in *Treponema* [257]. The membrane associated VWFA domains are similar to the extracellular adhesion molecule, thrombospondin-related anonymous protein (TRAP), found in malarial protozoan *Plasmodium* [258]. TRAP has been shown to bind mammalian hepatocytes and heparin sulfate proteoglycans [259]. Therefore, as structurally similar to TRAP, it was expected that VWFA domain containing proteins in spirochetes are involved in bacterial adhesion to mammalian extracellular matrix [257]. In *B. burgdorferi*, four genes encoding for VWFA domain containing proteins were identified including *bb0172*, *bb0173*, *bb0175*, and *bb0325* [255, 257, 260]. Among these genes, *bb0172* was found to encode for an outer membrane protein of *B. burgdorferi* that binds to the human integrin α3β1, via its VWFA domain [51]. In regard to the other Borrelial VWFA domain containing proteins, BB0173 was shown to be an inner membrane protein with its VWFA domain exposed internally to the periplasmic space [261], with its expression was regulated by the presence of oxygen in the culture medium.

To date, several attempts have been made to generate a protective vaccine to prevent LD in humans. Nonetheless, they have been considered ineffective to confer protection against LD caused by various genospecies of *Borrelia*, due to the fact that the antigens of these vaccines show high heterogeneity when compared among different serotypes of spirochetes. In our laboratory, vaccine studies showed promising results when using a short peptide (PepB), derived from the highly conserved VWFA-domain containing protein BB0172 [51]. Small and collaborators showed that the BB0172 derived peptide confer protection in the murine model of LD when administered subcutaneously as a conjugate vaccine [79]. In addition, no histopathological signs were observed post-immunization in any of the examined tissues. Although the combined evidence supports the promise of PepB as an approach to Lyme vaccine development, there is a critical need to evaluate the therapeutic efficacy and safety of novel formulations that will improve protection in both humans and animals across different *Borrelia burgdorferi* sl pathogens in time and space.

Central hypothesis and specific aims

Based on the presented literature review, my central hypothesis is that implementing carrier and/or scaffolding molecules in conjugation with the vaccine candidate PepB, derived from BB0172, will increase the efficacy of the PepB vaccine formulation against Lyme disease in the murine model. The objective of this work is to evaluate a vaccine candidate derived from a highly-conserved *B. burgdorferi* von Willebrand factor A domain containing protein, using different antigenic platforms. Therefore, our study is significant because it exploits the conserved nature of BB0172 protein allowing the generation of vaccine candidates that will diversify alternatives to control the spreading of Lyme disease in the US.

The overall hypothesis was challenged through the completion of the following specific aims:

Aim I: Evaluate the immunogenicity and efficacy of the PepB formulated in a protein scaffold derived from *B. burgdorferi*.

Aim II: Evaluate protective efficacy of the CRM197 and Tetanus toxoid heavy chain conjugated PepB in the murine model for Lyme disease.

Aim III: Evaluate protection of the optimal antigen formulation in mice via natural infection.

CHAPTER II

EVALUATION OF IMMUNOGENICITY AND EFFICACY OF THE PEPB ANTIGEN FORMULATED IN A PROTEIN SCAFFOLD DERIVED FROM *Borrelia burgdorferi*

Introduction

Protein scaffold is an alternative method used to generate immunogenicity of poorly or non-immunogenic antigens [262-264]. Naturally synthesized proteins with rigid, well-defined structure and intrinsic stability are considered effective scaffold that can be used for antigen presentation [265-267]. Some studies revealed that inserted epitopes into an unrelated scaffold protein structure was a feasible strategy to present the HIV-1 gp41 epitopes 2F5 and 4E10. In such experiments, author observed that after immunization, the scaffolded antigens produced significant high-binding affinity antibodies compared to the epitope peptide alone [264, 268]. Suitable scaffolds are molecules with sufficiently large regions at the molecular surface that are tolerant to substitutions or insertions without interrupting the integrity of the overall three-dimensional structure [269]. Also, scaffolds are considered efficient immunogens in many respects to other antigen presentation platforms, including peptide conjugates [270].

Reverse vaccinology has enabled us to determine and define the antigenic map of various pathogens, and structural vaccinology, which tremendously assist in identifying the 3-dimensional structure of pathogen-specific components, and potentially recognize

pathogen-specific antigens as well [271]. Therefore, the epitope-based vaccination can be used to mainly target critical neutralizing epitopes, and avoid inducing non-protective antibodies in vaccinated individuals [272].

There are a number of scaffolding alternatives such as Virus-Like Particles (VLPs) and backbone grafting. In the first case, virus-like particles (VLPs) have been used as vaccine development platforms for years. Furthermore, VLP scaffolds have been used to present immunogenic antigens from various viral, bacterial, or parasitic pathogens, known as chimeric VLPs [273]. Viral capsid proteins can be synthesized *in vitro* through exploiting the advancing biotechnology, and creating self-assembled, empty virus-like particles. In addition, these particles can be used as an adjuvant to boost the immune response [274-276]. Furthermore, VLPs can be presented by Antigen Presenting Cells (APC) and stimulate B and T- lymphocytes. Therefore, scientists have taken advantage of these immunogenic viral particles and used them as platforms to display different antigens with the objective of inducing a strong immune response [275].

Backbone grafting is a strategy that implies using protein scaffolds as a presentation platform, in which a protein backbone can be engrafted with different epitopes [264, 277, 278]. Correia and collaborators [3] showed that a viral epitope, derived from respiratory syncytial virus and inserted in a protein scaffold based structure was able to elicit potent neutralizing antibodies [279].

Borrelia burgdorferi has a single peptide transport system of the ABC transporters family, which resembles oligopeptide permease (Opp) and dipeptide

permease (Dpp) transport systems that are found in other bacteria [280, 281]. BBA34 is a 61 kDa surface lipoprotein, and one of 5 oligopeptide permease A homologs in *B. burgdorferi* and is denoted as OppA5 [60]. Furthermore, BBA34 participates in the transport of solutes like acetate and bicarbonate. Moreover, BBA34 is a lipoprotein exposed to the periplasmic environment of the *B. burgdorferi*, and the *bba34* gene is located on linear plasmid 54 (lp54). In addition, BBA34 is upregulated when the spirochetes are growing under tick feeding and mammalian host conditions [282]. In previous studies, Dr. Esteve-Gasent was able to demonstrate the immunogenicity of recombinant BBA34 lacking the lipidation target (personal communication, data not published). In addition to previous data from Dr. Esteve-Gasent, a former laboratory member, Dr. Christina Brock demonstrated the immunogenicity of BBA34:PepB in the murine model of Lyme diseases (Brock, C. PhD Thesis).

Based on the evidences described above, the borrelial membrane protein BBA34 was considered as a potential protein scaffolding molecule. Consequently, we hypothesized that by engrafting PepB into an exposed region of the borrelial protein BBA34, known as solute binding domain, we will be able to enhance the presentation of the PepB to the immune system, and therefore stimulate the generation of protective specific antibodies against *B. burgdorferi*.

Materials and methods

Cloning and expression of scaffolded antigen

The scaffolded construct BBA34:PepB (BP) was synthesized in the expression vector pET23a by Genscript (Piscataway, NJ) with optimized codon usage for expression in *Escherichia coli* . The plasmid was transformed into Rosetta™ (DE3) pLysS *E. coli* strain (Novagen, Madison, WI). Clones were verified by PCR and protein expression, and kept at -80°C for further use. Transformed cells were recuperated in LB medium containing appropriate antibiotics, and overexpression of BP was done at 37ºC using 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 hours. After overexpression, cells were harvested and lysed via sonication in lysis buffer (20 mM imidazole; pH 7.4) with addition of the protease inhibitor cocktail, HALT™ (Thermo Scientific, Inc.). The lysed cells were centrifuged, and the supernatant was run through affinity purification column using a His60 Ni Superflow resin (Clontech, Mountain View, CA) following manufacturer's recommendations. Following binding of proteins, the affinity column was washed with 30ml of wash buffer (40 mM imidazole; pH 7.4). Finally, the bound 6×His-tagged proteins was eluted with elution buffer (300 mM imidazole; pH 7.4), and the collected protein fractions were aliquoted and stored at -80ºC. Samples were separated and analyzed under SDS-12.5%PAGE conditions.

Purification of BBA34:PepB (BP) construct

Fractions containing the highest concentration of protein were pooled and concentrated using Spin-X™ centrifugal filters (Corning, Lowell, MA) with a 10 kDa MWCO. Then, concentrated proteins were passed through a column of Sephadex G-75 resin for size exclusion chromatography. Pierce™ BCA Protein Assay (Thermo Scientific, Inc., Rockford, IL) was used to quantify protein concentration of the collected fractions. Then, fractions with relatively high concentration were pooled and centrifuged as previously described, and applied to a PD-10 desalting column (GE Healthcare, Piscataway, NJ). Collected fractions were analyzed using SDS-12.5% PAGE, and fractions with highest concentration of protein were pooled and concentrated using the Spin-X centrifugal filters. A 61.5-KDa BBA34:PepB was purified and stored at 4°C until further use in animal experiments.

Expression and purification of control proteins

Control proteins OspC and BBA34 were previously cloned into pET23a and transformed into Rosetta™ (DE3) pLysS *Escherichia coli* (Novagen, Madison, WI) by Dr. Esteve-Gasent, and stored at -80ºC for further use. Transformed cells were recuperated in LB medium containing appropriate antibiotics, and overexpression of BBA34:PepB was done at 37ºC using 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 hours. After overexpression, cells were harvested, washed, and sonicated for a total sonication time of 10 minutes in lysis buffer (20 mM imidazole; pH 7.4) with addition of the protease inhibitor cocktail, HALT™ (Thermo Scientific, Inc., Rockford IL). The lysed cells were centrifuged, and the supernatants were applied to a His60 Ni

Superflow resin (Clontech, Mountain View, CA), for affinity purification according to the manufacturer's protocol. After binding, proteins were washed with 30ml of wash buffer (40 mM imidazole; pH 7.4). Finally, 6×His-tagged proteins were eluted with elution buffer (300 mM imidazole; pH 7.4), and the collected fractions were then analyzed using SDS–PAGE.

Purification of the control proteins was started with dialysis of the concentrated fractions of protein into dialysis buffer (50 mM Sodium Phosphate, 300 mM Sodium Chloride, 0.1% Triton X-100; pH 7.4) using Slide-A-Lyzer dialysis cassettes (Thermo Scientific, Inc., Rockford, IL). Dialyzed proteins were then concentrated with a 10 kDa MWCO (EMD Millipore, Billerica, MA). After that, control proteins were quantified using Pierce™ BCA Protein Assay (Thermo Scientific, Inc., Rockford, IL), and then were kept at 4°C until further use.

Immunization

Six to eight weeks old C3H/HeN mice (n=6 per group) were immunized subcutaneously with decreasing antigen dosages of 50µg/mouse for priming followed by 10µg/mouse at 14-days post-priming, and a final booster dose of 5µg/mouse at 28-days post-priming in combination with 5% Adjuplex™ adjuvant (Advanced BioAdjuvants LLC). In this study we included the following groups: BP, BBA34, OspC and a naïve control group. Eight weeks post-priming blood samples were collected, and specific IgG titers were evaluated by using ELISA methods previously described by others [79].

Infection

To evaluate protection induced by the scaffolded antigen BP, we infected mice (n=6) subcutaneously ten weeks post-priming with a dose of 10^5 Borrelial cells per mouse. First, PCR screening was conducted to detect the presence of essential plasmids required for the survival and infection of *B. burgdorferi* strain that was used in the challenge studies. The primers for PCR screening method were previously described [283]. Four weeks post-challenge mice were euthanized and blood and tissue samples were collected to evaluate protection. The sampled tissues included skin, spleen, inguinal lymph nodes, heart, bladder, and tibiotarsal joint. Tissues were cultured in BSK-II media complemented with 6% inactivated naïve rabbit serum and incubated at 32ºC and 1% CO_2. The cultures were blindly passed into fresh BSK-II media after 5 days post-initial inoculation and then incubated at 32ºC and 1% CO_2 for up to 21 days. The initial and blind passaged cultures were evaluated under dark field microscopy at 14 and 21 days post-inoculation for bacterial growth [284]. We evaluated bacterial burden in skin, spleen, inguinal lymph node, and tibiotarsal joint by qPCR as previously described [285].

Statistical Analysis

Antibody titers were analyzed with Two-way ANOVA to evaluate the significant differences in between the different immunized groups. In quantitative real time PCR data, Mann Whitney U test was run to statistically compare in between groups. All graphs and analysis were done using Prism 7.0 (GraphPad Software, Inc.).

Results

Plasmid profile of B. burgdorferi

The results of PCR screening showed that all essential plasmids were detected in the *B. burgdorferi* B31 strain that was used in challenge protocol to confirm the ability of that strain to colonize and induce infection in a host (Figure 2.1).

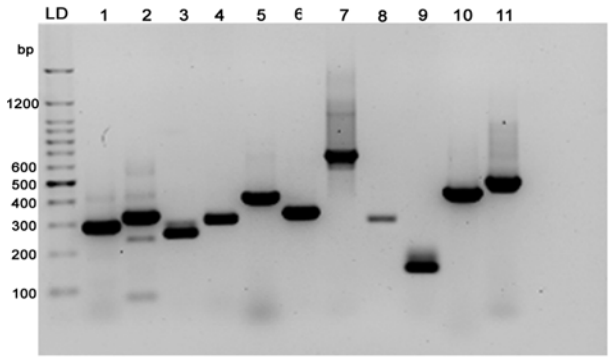

Figure 2.1: Plasmid profile of *Borrelia burgdorferi* B31 strain. PCR screening on 1% agarose gel revealed presence of all essential plasmids. Ld: DNA Ladder (LD) weight is represented in base pairs (bp) on the left of the image. 1: cp26; 2: lp5; 3: lp17; 4: lp25; 5: lp28-1; 6: lp28-2; 7: lp28-3; 8: lp28-4; 9: lp36; 10: lp38; 11: lp56.

Efficacy of BBA34:PepB construct

In previous studies, Dr. Christina Brock demonstrated that the immunization schedule described above was able to induce specific IgG antibodies in immunized animals using BBA34:PepB. Furthermore, Dr. Brock demonstrated the presence of comparable antibody titers in both BBA34 and BP immunized mice. Hence, in this study we evaluated the presence of protective antibodies in the immunized animals. To this end, we infected both control and immunized groups with 10^5 spirochetes/mouse via needle inoculation. As shown in Figure 2.2 immunized animals were able to induce high

specific antibody titers 8-weeks post-priming. In addition, all groups developed specific antibodies against *B. burgdorferi* post-challenge.

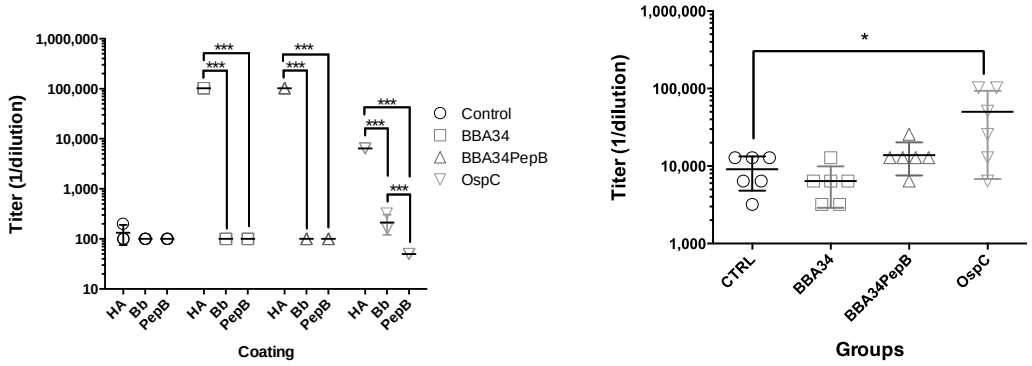

Figure 2.2: BBA34, BP and OspC induce specific antibodies in immunized C3H/HeN mice. (Left diagram) Specific IgG titers in the immunized groups. HA denotes Homologous Antigen; Bb denotes *Borrelia burgdorferi* whole cell lysates; PepB denotes the specific peptide B antigen used as coating agents in the ELISA. **(Right diagram)** anti-*Borrelia burgdorferi* IgG levels in the different groups 4 weeks post challenge. * Denotes significant differences: * *P* value < 0.05; *** *P* value < 0.001.

On the other hand, and as shown in Figure 2.3 immunized mice were not able to clear the infection when tissues were inoculated in BSK-II medium and incubated at 32ºC and 1%CO_2. As shown in this figure, even though there is significantly lower bacteria recovery from tissues isolated from immunized animals, most tissues were able to grow viable bacteria in media, and therefore, we considered that immunized animals were not protected.

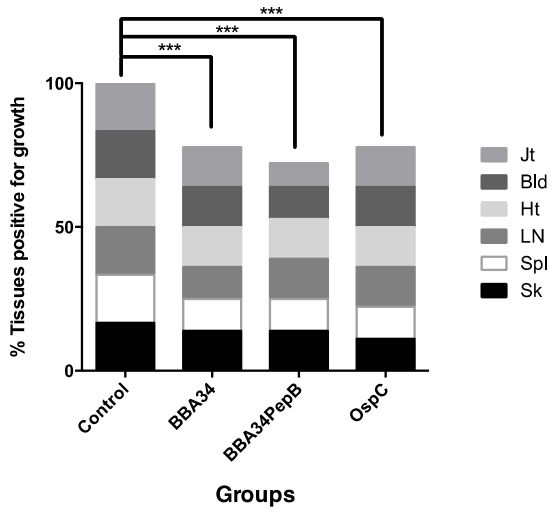

Figure 2.3. Bacterial recovery. In BSK-II medium complemented with 6% inactivated normal rabbit serum and incubated at 32ºC and 1% CO_2. In vaccinated groups 1/6 animals showed clean tissues. In BBA34:PepB immunized animals 2/6 spleens and 3/6 joints showed clearance. * Denotes significant differences: * *P* value < 0.05; *** *P* value < 0.001.

Furthermore, we evaluated the bacterial burden in the collected tissues by means of qPCR. As shown in Figure 2.4, there was significantly low bacterial burden in all examined tissues of BBA34:PepB compared with the control group. Interestingly, BBA34 and OspC groups generally had lower bacterial load in all tissues compared with the control group and the BP group. Taken together, and considering both bacterial burden and bacterial recovery from tissues, immunization with BP was not able to clear the infection in this experiment, and consequently we did not consider this alternative as a potential vaccine formulation in subsequent studies.

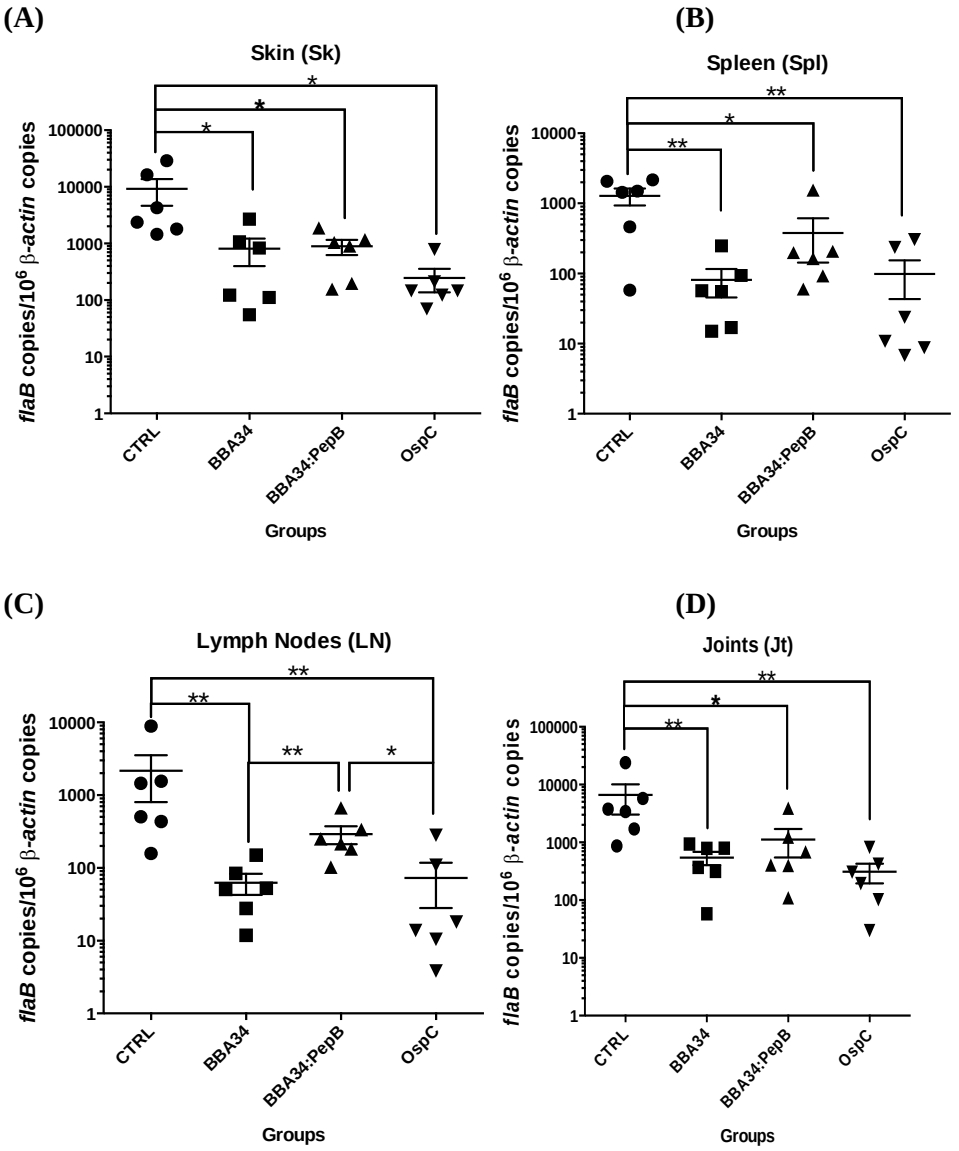

Figure 2.4. BBA34:PepB did not confer protection in immunized mice infected with *B. burgdorferi*. Bacterial burden was significantly reduced in mice immunized with BBA34:PepB, BBA34, and OspC in all examined tissues including skin **(A),** spleen **(B),** lymph nodes **(C)**, and joints **(D)** compared with control group. * Denotes statistical significances (* *P* value < 0.05; ** *P* value < 0.01) when compared with control group.

Discussion

The increasing of geographic distribution and prevalence of LD cases has been associated with expanding of public health concern since the disease was identified. Vaccination is an effective approach that can be used efficiently to control the LD cases in humans and reduce the economic burden of the disease [286]. Many studies have evaluated several vaccine candidates against LD including outer surface lipoproteins and other antigens [287, 288]. In our studies, PepB has shown promising results as a vaccine candidate against LD, providing 50% protective efficacy in tick challenge model when the peptide was conjugated to KLH [79].

Scaffolding molecule BBA34 in conjunction with PepB was used in this study to enhance the efficacy of the selected peptide in the murine model of LD. The use of scaffold protein BBA34 excludes introducing foreign immunostimulants that might induce adverse reactions, when they are tested in a host. Evaluation of immune response in immunized mice revealed presence of significant high antibody titers compared to the control group, however, PepB specific antibody titer was very low. Therefore, it may be revealed that the immune response was not enhanced against the PepB, this might be due to the fact that the scaffolding molecule impeded the appropriate exposure of the peptide to the immune system. The scaffolding molecule with inserted PepB might require additional optimization in order to be effective. This outcome is similar to the study of McLellan et al. in which authors showed that the use of scaffolding structure was unable to induce antibody response in immunized mice against an antigen derived from respiratory syncytial virus (RSV) [289].

Bacterial burden in tissues was evaluated to determine a protective efficiency of the scaffolded PepB antigen in immunized mice. The BBA34:PepB construct, and the BBA34 and OspC proteins showed a reduction in the bacterial load in the examined tissues compared with the control group. In addition, viable spirochetes were grown in the cultured tissues in BSK-II media from all immunized groups. Hence, the PepB was not considered protective in the scaffolding context, and other alternatives could be investigated to formulate an efficient PepB–based vaccine candidate. The 12-mer peptide (PepB) is unable to induce an immune response by itself, because of its small size, therefore it is essential to increase its size to render it immunogenic. The PepB design can be further explored in the context of a multiple-copy peptide antigen approach. In fact, multiple-copy peptide method has been shown that it stimulated a better antibody response than that seen with a single copy peptide conjugated with KLH [290].

In the multiple-copy peptide design the selected antigen is exposed in a well-defined orientation, and it excludes any unnecessary structural components that may include suppressor epitopes that might be found in scaffolding, and/or carrier molecules. In addition, a multiple-copy peptide is considered highly immunogenic, and can induce both humoral and cellular immune responses against HIV infection in human [291]. Furthermore, previous studies showed that a multiple-copy peptide antigen derived from the circumsporozoite protein (CSP) of *Plasmodium yoelii* was able to confer protection in mice against malaria infection [292]. Also, other antigen delivery systems can be used with PepB to improve its protective efficacy such as use of liposomes, archaeosomes,

polymersomes, and immuno-stimulating complexes (ISCOMs) [293-295]. These methods can be used to improve antigen persistence, uptake, and presentation, and thus able to potentiate immune response effectively.

In this study, the scaffolding of PepB approach did not provided with promising results, and therefore, we did not proceed with it. Hence, other novel methods were explored in terms of reformulation of PepB design, and/or conjugation with carrier molecules to improve the immunogenicity and protective efficacy of the PepB-based vaccine candidate.

CHAPTER III

EVALUATE PROTECTIVE EFFICACY OF THE CONJUGATED PEPB

Introduction

Lyme disease (LD) is a vector-borne disease transmitted by hard ticks of genus *Ixodes* spp. The prevalence of *B. burgdorferi* in infected ticks can reach up to 50% in endemic areas [296-298]. *Borrelia* genus is divided into two major clades, one group of species causing Lyme Disease (LD), and the other group causing relapsing fever. As mentioned in chapter 1, LD is the most prevalent tick-borne infection in the United States with more than 30,000 cases reported annually according to the Centers for Disease Control and Prevention (CDC). However, the CDC estimates that only 10% of all LD cases are actually reported, and thus, the actual infected cases will be closer to 300,000 per year [299, 300]. LD infection is transmitted to human and other animals via the bite of an infected tick, and with the geographical expansion of tick population in recent decades, primarily due to climate change, the risk for human infection is increased in the newly established tick habitats [301-303]. These events have been associated with a steadily increased numbers of notifiable tick-borne diseases, as well as the identification of new arthropod-borne pathogens such as *Borrelia mayonii* and *B. hermsii* [304].

Currently, there is no available vaccine against LD in humans, in addition, control measures are based on preventive exposure of individuals to infected ticks by wearing long sleeves and pants, avoiding tick infested area, and use insect repellents.

LYMErix (SmithKline Beecham) was the only available OspA-based vaccine for human use in the US, but it was withdrawn from the market in 2002. Hence, researchers focus their efforts to develop potential vaccine candidates against LD in human, using a number of different antigens such as OspA, OspB, OspC, decorin binding protein A (DbpA), BBK32, and RevA [305-307]. To date, several attempts have been made to generate a protective vaccine for use in humans. Nonetheless, they have been considered ineffective to confer protection against LD caused by various genospecies of spirochetes, due to high heterogeneity observed in such antigens within different serotypes of spirochetes.

Historically, the development of a LD vaccine has been based on modifications of either the outer surface protein A (OspA) or C (OspC). In fact, LYMErix by SmithKline Beecham, the first and only LD vaccine licensed for human use, was an OspA vaccine [69]. It was withdrawn from the market in 2002 due to lack of interest and potential side effects associated with using LYMErix. This vaccine formulation targeted the Borrelial OspA lipoprotein, which is expressed when the pathogen is in the arthropod tick vector. When expressed, OspA mediates the attachment of spirochetes to the tick mid-gut via its receptor (TROSPA) [144]. Homologs of TROSPA have been identified not only in *Ixodes scapularis* (vector of *B. burgdorferi* in the US), but also in *I. ricinus*, the tick species transmitting the infection in Europe [25], and *I. persulcatus*, the species transmitting *Borrelia* in Asia [26].

During tick feeding *B. burgdorferi* migrated to the salivary glands of ticks, and then to the mammalian host. That migration is facilitated by the downregulation of OspA

upon blood intake by the tick [22, 144]. Thus, OspA based vaccines are effective in conferring protective immunity by blocking the transmission of *B. burgdorferi* from the tick to the mammalian host [145]. In fact, the antibodies induced by the OspA vaccine neutralizes *B. burgdorferi* in the tick vector rather than in mammalian host. Furthermore, administration of OspA formulated vaccine to small rodents (reservoir hosts) has been used to reduce the prevalence of *B. burgdorferi* in the mammalian reservoirs and the tick vectors, and break the enzootic cycle of the disease in nature, that might reduce the incidence of *B. burgdorferi* infection in human [146, 147]. In addition, in veterinary medicine, multiple OspA based vaccines have been used to immunize dogs against LD, such as (Nobivac® Lyme by Merck Animal Health, Merck & Co. Inc.; LymeVax® from Zoetis and Recombitek® Lyme from Merial Inc.) [75, 148]. Nevertheless, to have an effective vaccine, specific antibodies have to neutralize *B. burgdorferi* while in the tick vector. Hence, individuals immunized with OspA vaccines need to maintain high antibody titers to be protected at all times, and thus, this can be one of the major drawbacks associated with using OspA-based vaccine.

The other important differentially expressed protein is the outer surface protein C (OspC). As described in Chapter 1, this lipoprotein is expressed during mammalian infection and it facilitates transmission of *Borrelia burgdorferi* sensu lato (s.l.) from the tick vector to the mammalian hosts [70]. Furthermore, OspC shows significant heterogeneity within *B. burgdorferi* s.l. isolates [153]. Therefore, in order to use a protective vaccine, any construct of OspC based vaccine is required to incorporate the heterologous potentially protective epitopes from different *B. burgdorferi* s.l. isolates. In

fact, to generate a complete and broadly protective vaccine against LD using the OspC antigen, it would require the use of 34 distinct epitopes [154]. Based on such observations, an octavalent and chimeric OspC vaccine candidate has been evaluated [155], and a recombinant, heptavalent LD vaccine has been developed by Zoetis Services LLC, VANGUARD® crLyme, formulated to prevent LD in dogs [156].

In our laboratory, vaccine studies showed promising results when using a short peptide (PepB), derived from a highly conserved protein (BB0172) present on the outer membrane of *B. burgdorferi* s.l. [51]. Basically, BB0172 is an extracellular exposed proteins, that binds to the human integrin $\alpha_3\beta_1$, via its von Willebrand factor A (vWFA) domain [51]. It is expressed only when the growth conditions of *B. burgdorferi* cultures are shifting from the room temperature pH of 7.6 (unfed tick conditions) to 37ºC pH 6.8 (fed tick conditions). In addition, *B. burgdorferi* is not expressing BB0172 in cultures adapted to either conditions. Furthermore, BB0172 is not detected by serum from infected animals; therefore, this formulation will be beneficial in differentiating infected animals from vaccinated ones (DIVA). Moreover, BB0172 is highly conserved among the different genotypes within the *B. burgdorferi* sensu lato complex present in the US and Europe. In an previous study, Small and collaborators [79] showed that the BB0172 derived peptide B (PepB) conjugated to the Keyhole Limpet Hemocyanin (KLH) hapten, confer protection in the murine model of LD. BB0172 is a chromosomally encoded membrane protein, that is highly conserved among pathogenic *Borrelia* spp. Even though PepB was able to induce high specific antibody titers in immunized animals and could protect when infecting with low doses of the pathogen, it was not protective at

high dose challenge with 10^5 spirochetes/mouse, and was partially protective when using the tick model of infection.

Taking together, in our study the BB0172 derived peptide PepB was conjugated to carrier molecules, which include cross reactive-material 197 (CRM197) and Tetanus Toxoid heavy chain (TTHc), with the objective to further improve its immunogenicity and long-term protection. As described in Chapter 1, the carrier proteins selected for this study, have been successfully used in conjugate vaccine to immunize millions of people around the globe. Of these, CRM197 and TTHc are common carrier proteins used mainly in Glycoconjugate vaccines [223], and also used in conjugation with peptide antigens such as Improvest® product (Pfizer Animal Health) [308]. The extensive body of information about their immunogenicity and safety profile in clinical use is well-known [309].

Overall, in the current study, the BB0172 derived peptide PepB was conjugated to carrier proteins including TTHc and CRM197. We immunized C3H/HeN mice with these antigens following prime-boost immunization protocol. The aim of our study is to evaluate the safety and protective efficacy of conjugated PepB formulations in the murine model of LD following needle inoculation with *B. burgdorferi*.

Materials and methods

Ethics statement

All animal related procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University, Animal Use Protocol number 2017-0022. The mice were kept in Texas A&M University animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Peptide design and conjugation

The selected antigen, a short peptide (Pep B) derived from the Borrelial outer membrane protein BB0172 was synthetized by Peptide 2.0 (Chantilly, VA) at a 95-98% purity. After synthesis, Pep B was conjugated to commercially available carrier molecules TTHc and CRM197. The conjugation process was done in collaboration with Fina Biosolutions LLC (Rockville, MD). In addition, Pep B conjugated to Bovine Serum Albumin (BSA) was generated to evaluate antibody levels against PepB in traditional Enzyme Linked Immunosorbent Assays (ELISA).

Borrelia burgdorferi strains and growing conditions

Borrelia burgdorferi B31 A3 virulent strain was used in this study. The bacteria were grown at room temperature (RT) on BSK-II media pH 7.6 complemented with 6% inactivated rabbit serum (iNRS), to mimic the unfed tick conditions. The culture was incubated until it reached a cell density of $1\text{-}2\times10^7$ spirochetes/ml, and then subcultured

in BSK-II medium pH 6.8 plus 6% iNRS at 37ºC and 1% CO_2 in order to mimic the fed tick conditions. Cultures were ready when they reached a cell density of 3 to 5×10^7 spirochetes/ml. To prepare whole cell lysates antigen for ELISA, cells were harvested, washed three times with HBSS buffer (HyClone, Thermo Scientific Inc.), counted, and lysed using 0.1mm glass beads on a BeadRuptor 24 (Omni International, Inc.) at 5,000 rpm for 5 minutes. Lysates were stored at -20ºC until use to coat ELISA plates. For the needle infection experiment, *Bb* cultures were prepared as above. The bacterial cultures were shifted from RT/pH 7.6 to 32ºC/pH 6.8 and incubated until the cell density reached $3\text{-}5\times10^7$ spirochetes/ml. Then, the bacteria were harvested, washed twice with HBSS buffer, and resuspended in HBSS buffer with inactivated naïve rabbit serum (50:50, *v:v*). Cell suspensions were quantified and adjusted to the required infective dose.

Immunization protocol

Six to eight week old female C3H/HeN mice were divided into four groups with 9 mice each. Two groups of mice served as control groups receiving the unconjugated carrier molecules, Cross Reacting Material 197 (CRM197) for group 1 and Tetanus Toxoid heavy chain (TTHc) for group 2. The other two groups received conjugated peptide, CRM197:PepB and TTHc:PepB, groups 3 and 4 respectively. All groups were primed at 50μg of the antigen subcutaneously per animal with their respective antigen. Animals were boosted with 10 and 5 μg/animal subcutaneously at days 14 and 28 post-priming. All vaccine formulations were prepared in 5% Adjuplex™ (*v:v*, Sigma-Aldrich, St. Louis, MO). On week 8 post-priming, blood was collected from all groups to detect the generation of specific anti-PepB IgG levels. Three mice per each group were

euthanized and samples from skin, heart, liver, kidney, and tibiotarsal joint were collected to evaluate histopathological signs post-immunization. On week 12 post-priming, mice were challenged with pathogenic *B. burgdorferi* via subcutaneous inoculation.

B. burgdorferi challenge protocol

To evaluate protective efficacy of the formulated antigen, immunized mice (n=6) were challenged 12 weeks post-priming by administering an infectious dose of 10^5 *B. burgdorferi*/mouse (1000× the infectious dose 50 (ID_{50})) subcutaneously in HBSS containing inactivated rabbit serum (*v:v*, 50:50). Three weeks post-challenge, mice were euthanized and blood and tissue samples were collected from each mouse. The sampled tissues included skin, spleen, inguinal lymph nodes, heart, bladder, and tibiotarsal joint. Tissues were cultured in BSK-II media complemented with 6% inactivated naïve rabbit serum and incubated at 32ºC and 1% CO_2. The cultures were blindly passed into fresh BSK-II media after 5 days post initial inoculation and then incubated at 32ºC and 1% CO_2 for up to 21 days. The initial and blind passaged cultures were evaluated under dark field microscopy at 14 and 21 days post-inoculation for bacterial growth [284]. We evaluated bacterial burden in skin, spleen, inguinal lymph node, and tibiotarsal joint by qPCR as previously described [285]. Finally, parts of skin, heart, liver, kidney, and tibiotarsal joint were collected for histopathology.

Histopathology

Tissues were collected from immunized mice on week 8 post-priming, and 3 weeks after challenge. After that, they were preserved in 10% formalin, while tibiotarsal joints were decalcified in 10% EDTA for 4 weeks prior to further processing. All tissues were histologically processed and stained with H&E. The histopathologic changes in post-immunized and infected tissues were evaluated by a board-certified pathologist, and inflammation in selected tissues was scored from 0 – 4. Inflammation scores were chosen based on the severity of inflammation, starting from normal = 0 (no inflammation), minimal = 1 (one small foci of inflammation), mild = 2 (2-5 foci of inflammation with increased numbers of inflammatory cells), moderate = 3 (multifocal inflammation with significant number of inflammatory cells), and severe = 4 (multifocal to diffuse, with more than 30% of section infiltrated with inflammatory cells) [310].

Enzyme-linked Immunosorbent Assay

The enzyme-linked immunosorbent assay (ELISA) was used to evaluate IgG and IgM titers in serum samples from immunized animals, and from immunized and infected animals. In brief, 96 well Nunc Maxisorb® plates (Thermo Scientific, Ltd.) were coated overnight at 4ºC with Peptide B conjugated to Bovine Serum Albumin (BSA; Fina Biosolutions Inc. (Rockville, MD)) at a concentration of 100 ng/well. To evaluate antibody response to borrelial whole cell lysate, *B. burgdorferi* A3 strain shifted from RT/pH 7.6 to 37ºC/pH 6.8 as described above, was used to coat the plates at a cell density of 10^7 *Borrelia* cells/well. After coating with carbonate buffer pH 9.6, plates were washed three times with washing buffer (Phosphate Buffered Saline with 0.1%

Tween 20, (PBS–T)) and blocked with blocking buffer (PBS–T containing 3% BSA) for 1 hour at room temperature. Following three washes with PBS–T, mice sera were loaded to the wells in 2-fold serial dilutions in dilution buffer (PBS–T containing 1% BSA) in duplicate across the plate ranging from 1:100 to 1:102,400. Plates were incubated for 1 hour at room temperature, followed by three washes with PBS–T. Plates were subsequently incubated with secondary conjugated anti–mouse antibody (Horseradish Peroxidase, HRP) diluted 1:3,000 in dilution buffer. After washing, plates were developed using the substrate *o*-Phenylenediamine dihydrochloride (OPD) (Pierce, Thermo Scientific, Ltd), and read at the optical density (OD) of 450 nm by using the Bio-Tek Synergy™ H1 microplate reader and Gen5™ software (BioTek Instruments Inc., Winooski, VT).

Quantitative measurement of tissue spirochetes

To evaluate bacterial burden of spirochetes in infected mice, qPCR was performed. To this end, DNA was isolated from collected tissues (skin, spleen, lymph node and tibiotarsal joint) using the High Pure PCR Template Preparation Kit (Roche Applied Science, Indianapolis, IN). After measuring quality of DNA, to evaluate the presence of *B. burgdorferi*, the flagellin B (*flaB*) gene was amplified as previously described [311]. For reference, the murine β-actin gene was utilized for each sample. Final results were reported as *flaB* copies per 10^6 copies β-actin. All primers and amplification conditions have been described elsewhere [285, 312].

Complement Assay

Serum samples collected as described above (8 weeks post-priming) were used to evaluate whether immunized animals generated borrelicidal antibodies. To this end, *B. burgdorferi* was shifted from RT/pH 7.6 to 32ºC/pH 6.8 and cultured in the presence of 1% CO_2 to induce expression of BB0172 protein. After the cell density reached 2–5×10^7 Bb/ml, the culture was washed twice with HBSS buffer, and finally resuspended in BSK-II media with HBSS buffer (50:50, *v:v*). *B. burgdorferi* cells (10^6/well) were incubated with serial dilutions of sera from naïve and immunized animals in a 96-well plate for 1hr at 37ºC, followed by the addition of 1:10 dilution of commercially available rabbit complement (Sigma-Aldrich). The mixtures were incubated for an additional 1hr at 37ºC. Borrelial survival was measured before and after the addition of rabbit complement by doing 10-fold serial dilutions in fresh BSK-II media, and incubating the cells at 32ºC and 1% CO_2. Bacteria growth was evaluated under dark field microscopy at 48 and 72 hours.

Statistical Analysis

Antibody titers and presence of bactericidal antibodies were analyzed with Two-way ANOVA to evaluate the significant differences in between the diverse immunized groups. In quantitative real time PCR data, Kruskal-Wallis test was run to statistically compare between groups. All graphs and analysis were done using Prism 7.0 (GraphPad Software, Inc.).

Results

Efficacy of PepB conjugated vaccine candidate

Mice were immunized with conjugated peptide CRM197-PepB and TTHc-PepB according to previously described protocols [79]. In order to evaluate humoral immune response of immunized mice, sera from 3 animals of each group were collected at 8 weeks post-priming. To detect IgG and IgM titers, the PepB antigen was used to coat the ELISA plates. As shown in Figure 3.1, high IgG titers (100,000) were observed in all 3 animals immunized with conjugated PepB vaccines, compared to low titer of (100) in both carrier molecule immunized animals (CRM197 and TTHc groups). In regards to IgM levels, they were present at low titer (100) in all 3 animals in all conjugated PepB groups, and in the unconjugated versions of CRM197 and TTHc groups.

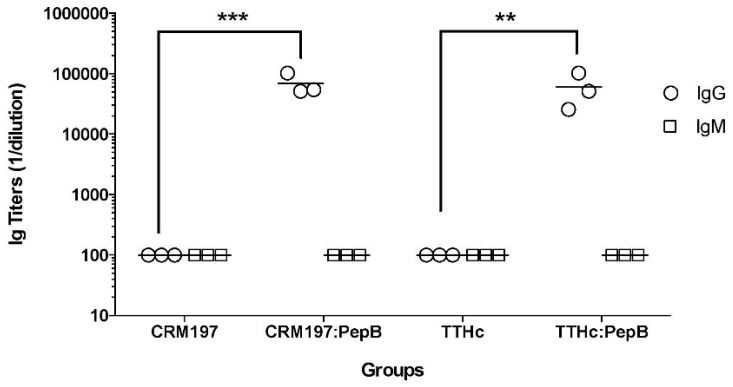

Figure 3.1. Peptide B specific antibodies. High IgG titers were detected at 8–weeks post–priming, while IgM titers of immunized animals were low at basal levels. * Denotes statistical significances (* *P* value < 0.05; ** *P* value < 0.01) when compared with control groups.

The immune response of animals after challenge via subcutaneous needle inoculation with 10^5 spirochetes/mouse ($1000\times ID_{50}$) was detected by tracking antibody titers in the serum samples which were collected at 3–weeks post–challenge. As shown in Figure 3.2A, ELISA plates coated with Pep B antigen showed significant high IgG titers around (1:10,000) in all immunized animals with conjugated PepB compared to the results observed in animals receiving the unconjugated CRM197 and TTHc, which were at basal levels (1:100). We used whole *Borrelia* lysate antigen to evaluate the development of anti-*Borrelia* antibodies post–challenge (Figure 3.2.B). ELISA results of IgG titer were generally high at around (1:10,000) in all immunized and infected animal groups, except one animal from TTHc:PepB group had low IgG titer of (1:100).

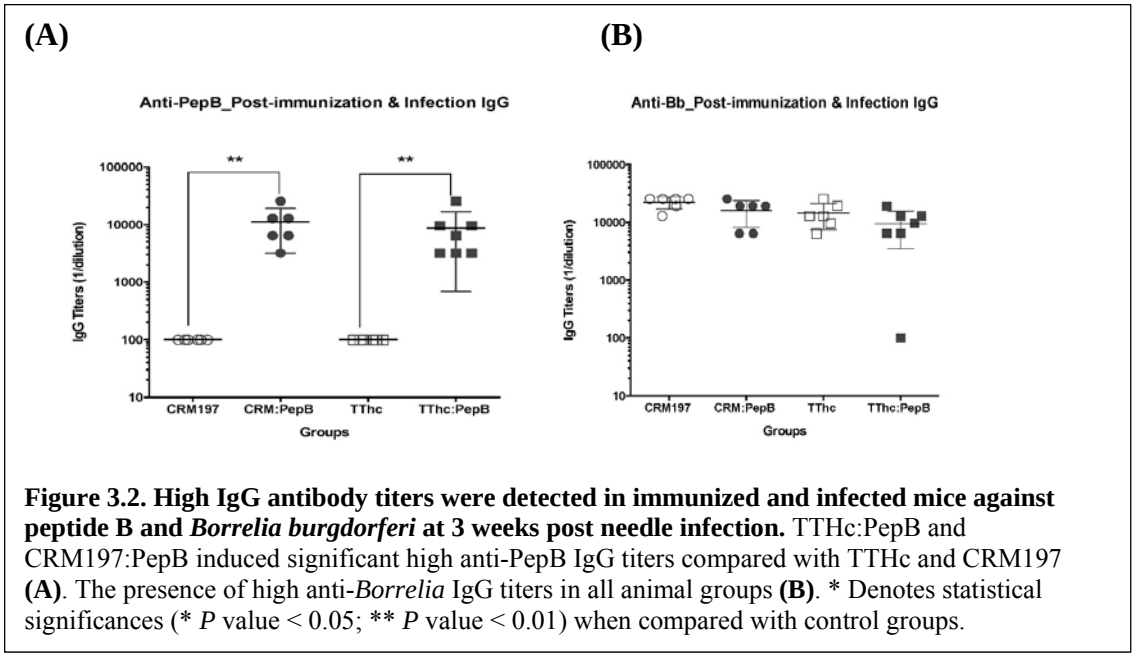

Figure 3.2. High IgG antibody titers were detected in immunized and infected mice against peptide B and *Borrelia burgdorferi* at 3 weeks post needle infection. TTHc:PepB and CRM197:PepB induced significant high anti-PepB IgG titers compared with TTHc and CRM197 **(A)**. The presence of high anti-*Borrelia* IgG titers in all animal groups **(B)**. * Denotes statistical significances (* P value < 0.05; ** P value < 0.01) when compared with control groups.

Evaluation of protective efficacy of the conjugated Pep B includes recovery of Lyme spirochetes from collected tissues of immunized animals post-challenge. Immunized C3H/HeN mice were infected at 12-weeks post-priming with subcutaneous inoculation of 10^5 spirochetes/mouse. Mice were euthanized at 4-weeks post-challenge, and bacterial burden in animal tissues including skin, spleen, lymph node, and tibiotarsal joints was determined by qPCR to evaluate protection in immunized animals. TTHc:PepB group showed significant reduction of spirochetes load in skin, lymph nodes, and joints compared to the TTHc group, which had high copy numbers of *flaB* (Figure 3.3). The experiment of immunizing mice with TTHc:PepB was repeated to confirm the observed results. As shown in Figure 3.4, the bacterial burden was significantly reduced in skin and spleen in TTHc:PepB immunized animals. However, in animals receiving the CRM197:PepB antigen, the spirochete load was relatively low compared to the CRM197 group in lymph nodes, and joints, but the significant reduction was only observed in skin samples .

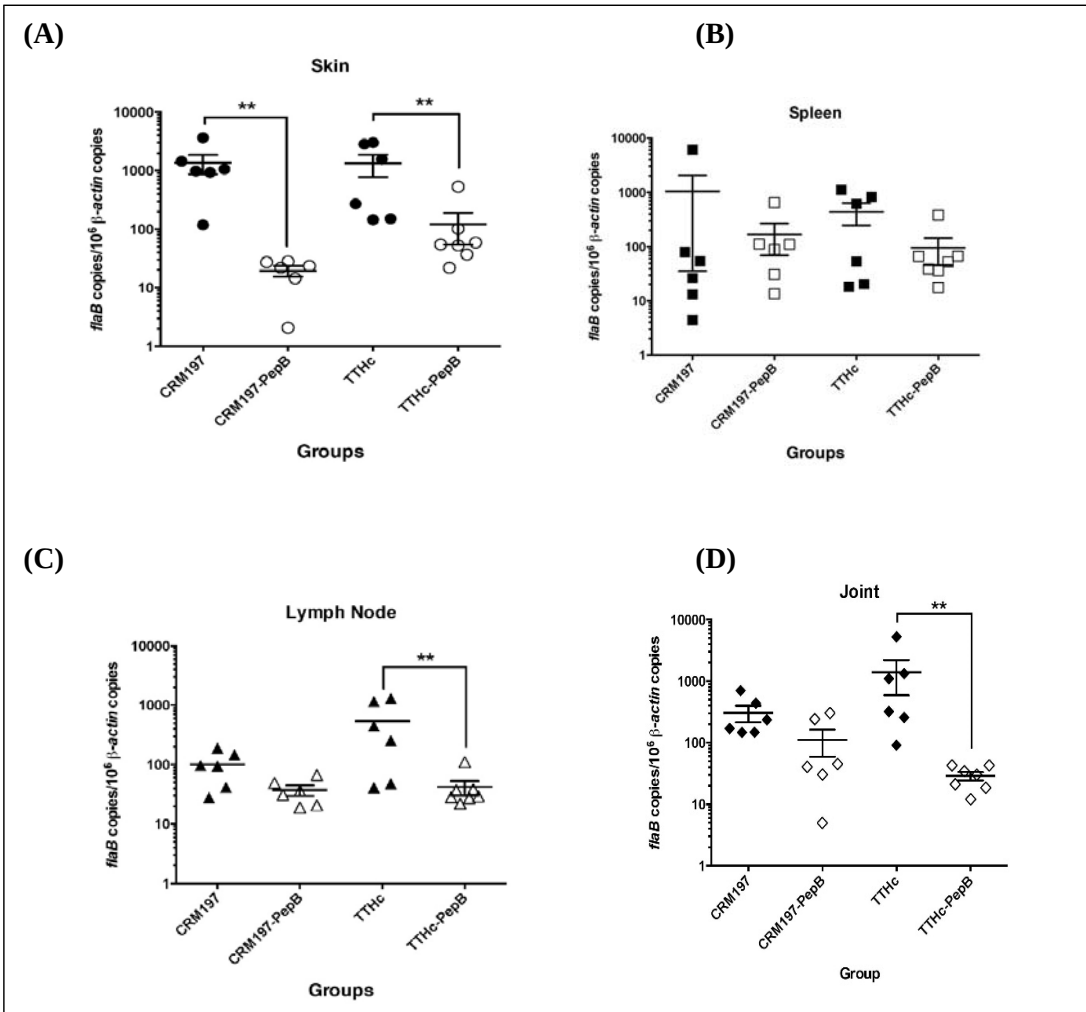

Figure 3.3. TTHc:PepB conferred protection in immunized mice infected with *B. burgdorferi*. Bacterial burden was significantly reduced in mice immunized with TTHc:PepB in all examined tissues including skin **(A)** lymph nodes **(C)**, and joints **(D)** when compared with TTHc immunized animals. CRM197:PepB showed reduction in bacterial load, but it was only significant reduction in skin **(A)** when compared to CRM197. * Denotes statistical significances (* *P* value < 0.05; ** *P* value < 0.01) when compared with control groups.

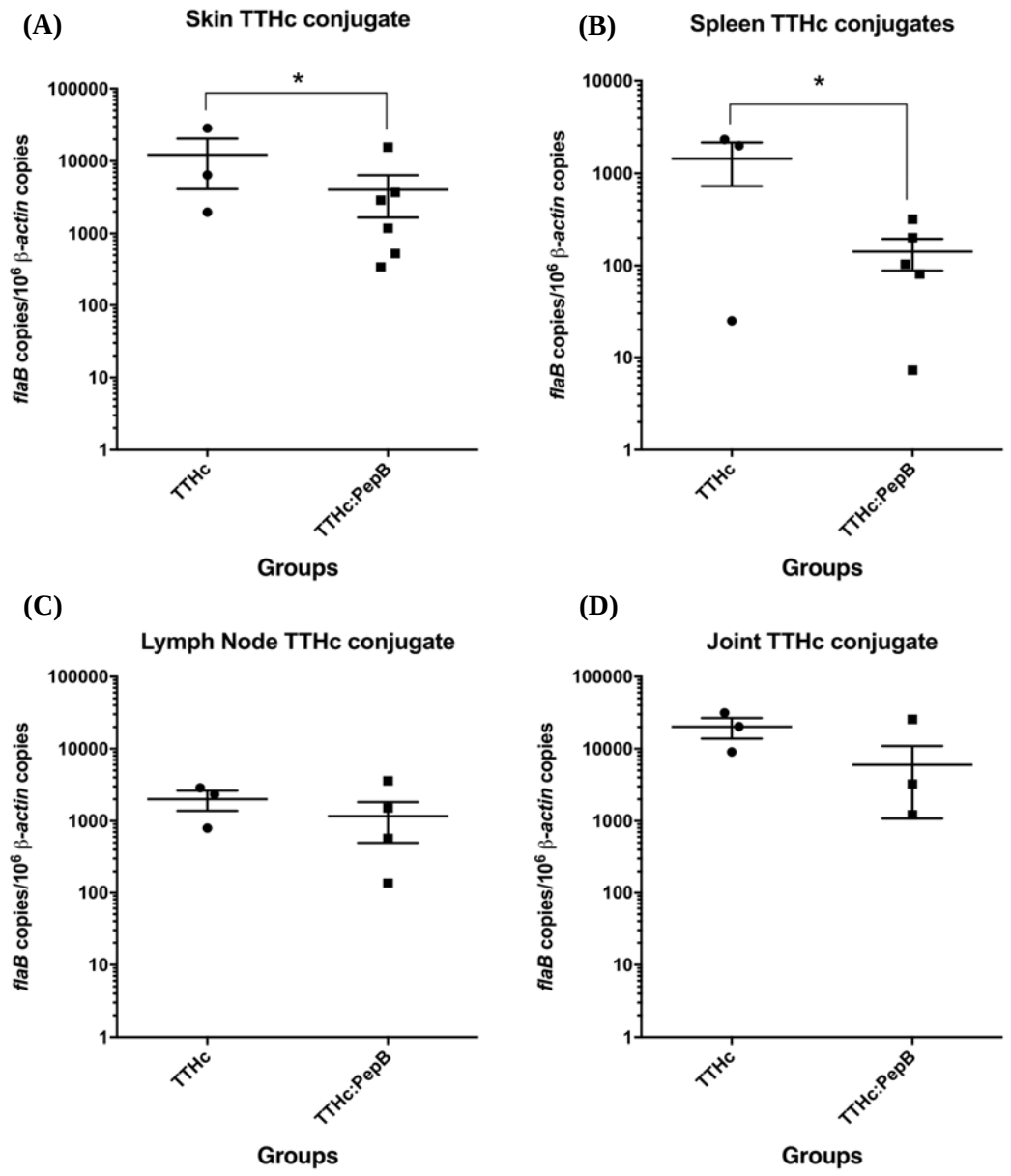

Figure 3.4. TTHc:PepB protection was replicated in a second experiment. Bacterial burden was significantly reduced in mice immunized with TTHc:PepB in some of the collected tissues including skin **(A)** and spleen **(B)** compared with TTHc. * Denotes statistical significances (* *P* value < 0.05; ** *P* value < 0.01) when compared with control groups.

Similarly, bacterial recovery was evaluated in different tissues by growth in BSK-II medium. *Borrelia burgdorferi* was recovered in culture from 2 out of 7 mice immunized with TTHc:PepB group, whereas *B. burgdorferi* was recovered from all tissues of all other immunized animals except in CRM197:PepB group, while *B. burgdorferi* was recovered only from 4 out of 6 cultures from skin sample (Table 3.1). In regard to TTHc: PepB group, this experiment was repeated to confirm our results, and it shown that *B. burgdorferi* was recovered in all sampled cultures only from 2 out of 5 mice (Table 3.2). Therefore, overall it was estimated that TTHc:PepB conferred 66% protection in immunized mice.

Table 3.1. Recovery of *B. burgdorferi* from sampled tissues of immunized mice at 3 weeks post needle infection (Biological replica 1)

Group/ 10^5 spirochetes/mouse	Skin	Lymph Node	Spleen	Bladder	Heart	Joint	Total
CRM197	6/6	6/6	6/6	6/6	6/6	6/6	36/36
CRM197:PepB	4/6	6/6	6/6	6/6	6/6	6/6	34/36
TTHc	6/6	6/6	6/6	6/6	6/6	6/6	36/36
TTHc:PepB	2/7	2/7	2/7	2/7	2/7	2/7	4/42

Table 3.2. Recovery of *B. burgdorferi* from sampled tissues of immunized mice at 3 weeks post needle infection (Biological replica 2)

Group/ 10^5 spirochetes/mouse	Skin	Lymph Node	Spleen	Bladder	Heart	Joint	Total
TTHc	3/3	3/3	3/3	3/3	3/3	3/3	18/18
TTHc:PepB	3/5	3/5	3/5	3/5	3/5	3/5	18/30

Evaluation of IgG isotypes to determine whether Th1 and /or Th2 immune response was induced in immunized animals, conjugated PepB groups, post-infection with *B. burgdorferi*, showed significant increase in IgG1 titers compared to the TTHc and CRM197 groups. Also, IgG2a titers showed the same trend as IgG1 (Figure 3.5).

Evaluate safety of administered antigens

The safety of TTHc:PepB and CRM197:PepB in immunized animals was evaluated at 8-weeks post-priming by histopathology. These antigens are considered safe when they are not inducing inflammation or other micro or macroscopical tissue damage post-inoculation. Conjugated PepB groups including CRM197:PepB and TTHc:PepB showed a slight inflammatory response in spleen, liver and joint, that seem to be correlated with protection against infection, principally in the TTHc:PepB immunized group. Overall, our experiments did not reveal significant inflammation in either group after immunization (Figure 3.6).

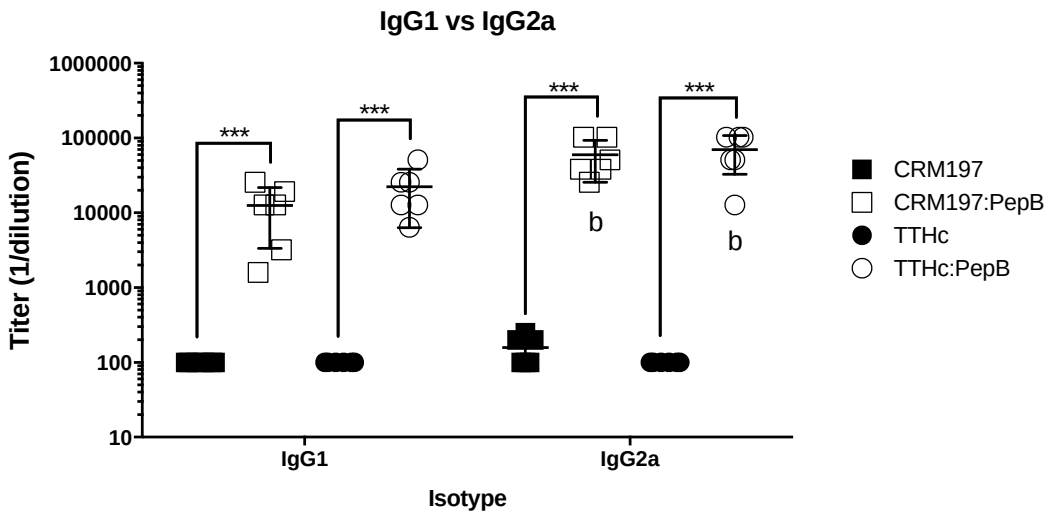

Figure 3.5. IgG1 and IgG2a antibody titers at 3 weeks post needle infection. * Denotes statistical significances (* *P* value < 0.05; ** *P* value < 0.01; *** *P* value < 0.001) when compared with control groups. Letter denotes statistical significance when comparing IgG1 and IgG2 levels (b denotes *P* value < 0.01).

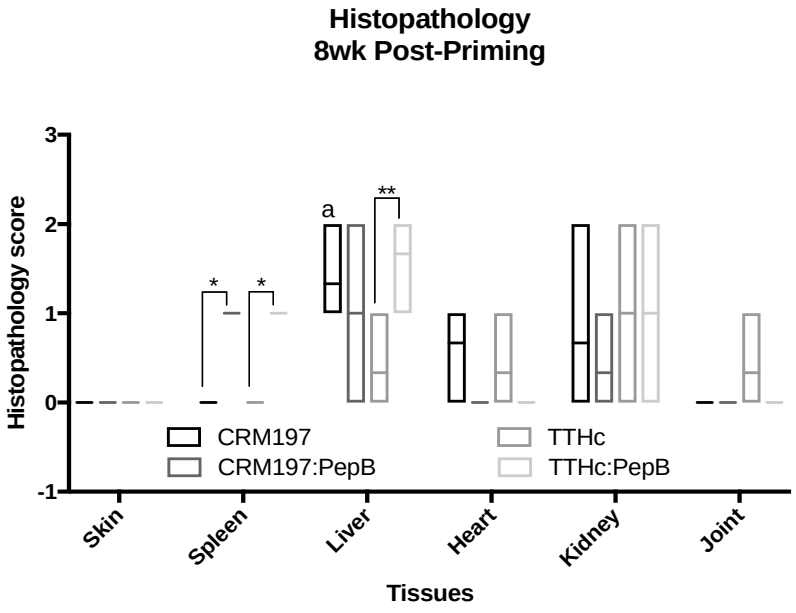

Figure 3.6. Histopathological changes score in immunized animals at 8 weeks post-priming. * Denotes statistical significances (* *P* value < 0.05; ** *P* value < 0.01) when compared with control groups.

Evaluate the serum bactericidal assay of immunized mice

The serum bactericidal assay was measured in sera of immunized mice using commercially available rabbit complement (Sigma-Aldrich). The antibodies from immunized sera in both constructs CRM197:PepB and TTHc:PepB were effective in reducing the number of *B. burgdorferi* cells at all tested serum dilutions, including (1:50, 1:100, 1:1000, and 1:2000). It was obvious that sera from the immunized mice with CRM197:PepB and TTHc:PepB constructs showed a significant reduction in borrelial cells compared to the negative group (Figure 3.7).

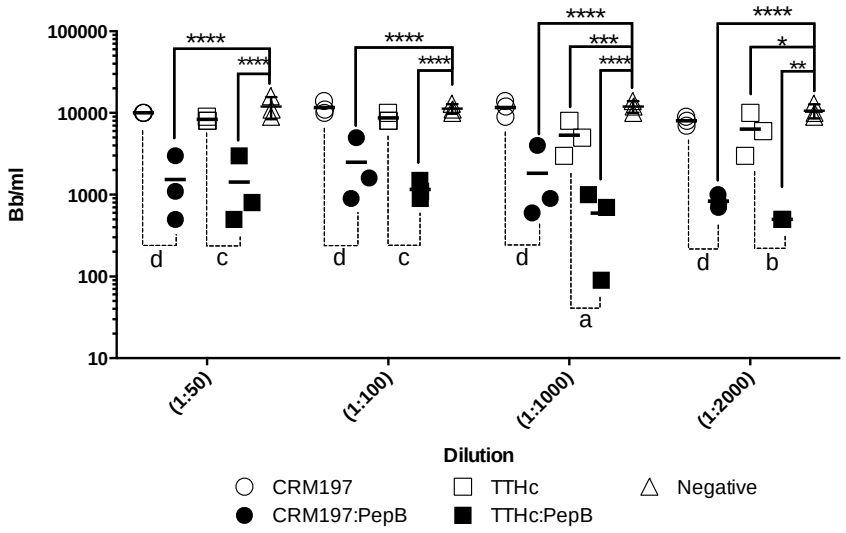

Figure 3.7. Anti-PepB antibodies are Borrelicidal. *B. burgdorferi* cells (10^6/ml) were incubated with serial dilutions of sera from naïve and immunized animals in a 96-well plate for 1hr at 37ºC, followed by the addition of 1:10 dilution of commercially available rabbit complement. The mixture was incubated for an additional 1hr at 37ºC. Borrelial survival was measured before and after the addition of rabbit complement by doing 10-fold serial dilutions in fresh BSK-II media, and incubating at 37ºC and 1% CO2. Bacteria growth was evaluated under dark field microscopy at 72 hours. Negative indicates no addition of immunized serum. 1:10, 1:25, 1:50 and 1:100 represent dilutions of the serum added. * Denotes statistical significance: (** P value < 0.01; **** P value < 0.0001). Letters denote statistical significance when comparing control groups with their corresponding PepB group (*a*: P value < 0.05; *b*: P value < 0.01; *c*: P value < 0.001; *d*: P value < 0.0001.

Discussion

Investigations into alternative antigens for LD vaccine in humans have been considered by researchers, since there is currently no availability for such vaccine in the markets. Many antigens have been tested as vaccine candidates to control LD infection [313]. However, most studies have been focused on outer surface proteins such as OspA and OspC [72, 75]. In addition, there are other few novel antigens that have been used as vaccine candidates such as Decorin-binding protein A (DbpA) [314, 315], and BBA52 [316]. Our team have previously identified BB0172, a chromosomally encoded protein, which is extracellularly exposed, that binds to the human integrin $\alpha_3\beta_1$, via its von Willebrand factor A (vWFA) domain [51]. Also, BB0172 derived peptide has been successfully used by our team as a vaccine candidate that was able to confer protection in mice challenged by needle inoculation with low doses of borrelial cells ($10\times$ ID_{50}) [79].

In this study, we explored the protective efficacy of the conjugated PepB following subcutaneous inoculation with 10^5 spirochetes/mouse, in which TTHc:PepB had the highest protective efficacy (66%) in immunized mice compared to CRM197:PepB immunized group. Based on the previous study [79], we sampled 3 animals from each group to evaluate antibody titers, and histopathological changes at 8-weeks post-priming. The humoral immune response is predominantly demonstrated to be responsible for protection against infection with *B. burgdorferi* [317-319]. Serum bactericidal assay (SBA) is mediated by antibody and complement reactions that lead to bacterial lysis. It is an effective indicator to evaluate functional activity of antibodies

[320]. In our study, we showed that conjugated peptide B was able to elicit production of borrelicidal antibodies. These findings are supported by other studies that showed the carrier proteins TTHc and CRM197 are used with licensed conjugate meningococcal vaccines in Europe, and they have shown to induce robust bactericidal antibodies [320-322]. Such antibodies were able to confer protection against septicemia and meningitis caused by different strains of *Neisseria meningitis* [323]. However, in these previous studies, the immune response was slightly skewed toward a Th1. In contrast, in our current study, the conjugated pepB formulations were able to elicit a mixed Th1/Th2 immune response, as determined by elevated IgG1 and IgG2a titers in immunized mice after needle infection. Based on previous publications, the predominance of Th1 immune response is important to control LD spirochetes in mice and humans [324-327].

In general, peptides have limited stimulation of B or T cells repertoire since they are small in size [328]. Therefore, different approaches have been implemented to improve peptides' efficacy such as novel folding strategies that are used to mimic the native structure of the selected epitopes [329], and other approaches that include, but not limited to, the use of scaffolding molecules, incorporation of additional immunostimulatory molecules, and the use of carrier proteins [330-332]. Of these strategies, the use of carrier molecules has been quite successful. In particular, the tetanus toxoid (TT) has been used extensively. Structurally, TT is composed of the light chain (LC) (catalytic domain), and the heavy chain (HC) (binding domain). The latter contains two functional domains; an amino-terminal portion (H_N) and a carboxy-terminal portion (H_C). The H_C fragment is the receptor binding domain, whereas H_N fragment is

responsible for LC translocation in the cytosol [333]. Tetanus toxin is a potent neurotoxin produced by anaerobic bacteria of *Clostridium tetani*, and it can be chemically detoxified to tetanus toxoid. This is used as a vaccine against the tetanus disease, and also as a carrier protein in conjugate vaccines [334]. We included TTHc in this study since it is commercially available, and its precursor (TT) has already been licensed and in the market. The immunogenicity of TT is attributed to the H_C domain, which is the principle component of TT molecule, as the toxin neutralizing epitopes are located within that domain [335, 336]. Furthermore, it contains potent T-cell universal epitopes, and the H_C domain is also responsible for binding and trafficking properties of TT [337, 338]. In fact, the immunogenicity of the H_C domain of TT was indicated when the recombinant form of that domain was able to confer protection in mice following challenge with tetanus toxin [339]. It has been shown that immunization of mice with OSP:rTTHc was able to produce bactericidal antibodies against *Vibrio cholerae* [340]. In addition, the TTHC has been shown to activate specific $CD4^+$T cells including TH1 cells [341, 342]. Taken together, synthetic peptides are considered a promising approach for the development of potential novel vaccines for an array of infectious diseases [343]. They also provide safe and cost-effective method that can be used alternatively to the conventional vaccine platforms [344].

In our study, the immune response showed a slight increase of IgG2a titers compared to IgG1 isotype in immunized mice at 3 weeks post-infection. That trend might enhance the efficacy of the conjugated PepB formulation to protect the immunized mice from the spirochetal challenge, especially in TTHc:PepB group that showed 70%

protection in immunized mice, through the evaluation of bacterial recovery from different animal tissues. Part of that mechanism might be attributed to the fact that murine IgG2a isotype was found to be more effective in serum bactericidal assay (SBA) than IgG1 isotype, when they were tested against *Neisseria meningitidis* strain. Furthermore, in the same study, it was shown that IgG2a was more efficient in opsonophagocytosis (OP) assay than IgG1 [345].

In addition, the difference in protective efficacy between the conjugated carriers in our study could be related to different number of PepB molecules loaded in each carrier molecule. In particular, each molecule of TTHc was conjugated to 12 PepB molecules, whereas CRM197 was only conjugated to 9 peptides. This explanation could be supported by studies that illustrated that hapten density in a carrier protein is a key factor for the induction of a protective immune response [346-349]. In addition, a hapten density also has an effect on the selectivity and breadth of the induced antibody repertoire, as it has been found that high hapten density in a conjugate was able to produce broader spectrum antibodies than low hapten density construct [350]. Moreover, when developing meningococcal C conjugated vaccines, it was concluded that TT had superior immunogenicity in GCMP-TT compared to GCMP-CRM197 vaccine [351, 352]. Also, TT has more thermal stability than CRM197, and it was found to retain its immunogenicity following exposure to high temperatures [353].

After needle challenge with *B. burgdorferi*, TTHc:PepB showed around 66% protective efficacy in immunized mice, whereas CRM197:PepB was not protective. Therefore, TTHc:PepB considered the most effective vaccine candidate formulation that

can be used for further studies. Thus, the current results suggest that TTHc:PepB is the most efficacious vaccine candidate and we proceeded with that conjugated pepB formulation to challenge immunized mice with the tick model of infection (discussed in the next chapter).

Taken together, our studies demonstrate that TTHc:PepB construct showed a high protective efficacy against *B. burgdorferi* infection compared to the other formulation. Thus, it is a promising vaccine candidate that will play a major role in the control of Lyme disease, with important DIVA feature (differentiate between infected and vaccinated animals).

CHAPTER IV

PROTECTION INDUCED BY TTHC:PEPB IN MICE USING THE NATURAL ROUTE OF INFECTION

Introduction

As mentioned in previous chapter, there is no vaccine available against LD for use in humans. Hence, prevention of LD relies upon control measures that limit exposure to potentially infected ticks, such as the wearing of long sleeves and pants, the avoidance of tick-infested areas, and the use of insect repellents.

Generally, *Borrelia* species lack classical virulence factors, instead they have the ability to survive within the infected hosts and evade their immune response by alterations in their outer surface proteins [354]. These spirochetes are also unable to synthesize many of essential amino acids, nucleotides and fatty acids needs due to lack of major biosynthetic pathways. Therefore, they rely on their vertebrate host and tick vector to meet their nutritional requirements [355, 356]. The changes in gene expression of spirochetes is the key role for their survival in two disparate environments, the tick vector and vertebrate host [357].

The outer surface proteins of *Borrelia burgdorferi* have been extensively targeted in vaccine studies especially OspA and OspC lipoproteins. These antigens are surface exposed and they have been shown to be immunogenic and can induce protective antibody responses [208, 358]. In addition, OspA and OspC have acquired the most attention as anti-Lyme vaccine candidates since these proteins are well characterized and

identified. Historically, the OspA based vaccine LYMErix™ was the only product that was licensed by the FDA, and distributed in the U.S. markets. However, in response to lack of public interest and associated side effects, it was withdrawn from the market in 2002.

Reverse vaccinology has enabled researchers to synthesize and test different potential vaccine candidates to induce protective immunity [359]. It provides information regarding the identification of surface-exposed antigens (epitopes) that can be tested in either *in vitro* or *in vivo* models to effectively screen these new candidates [360]. It is also a useful tool for recognizing novel vaccine candidates through the genome analyses of different isolates within the same species or between closely-related species [361, 362]. Through this technology, our team has identified a short peptide (PepB) that induced a protective immunity in mice against infection with *B. burgdorferi* [79]. PepB is derived from a highly conserved protein (BB0172), which is exposed extracellularly, and binds to the human integrin $\alpha_3\beta_1$ at its von Willebrand factor A (vWFA) domain. This protein is expressed when the growth conditions of *B. burgdorferi* cultures are shifted from a pH of 7.6 at room temperature (unfed tick conditions) to a pH of 6.8 at 37ºC (fed tick conditions). However, *B. burgdorferi* in cultures adapted to either condition do not express BB0172 [51]. In addition, BB0172-derived peptide has not been detected by serum in infected animals, making it potentially useful for differentiating infected animals from vaccinated ones (DIVA) [79]. Furthermore, among the different genotypes in the *B. burgdorferi* sensu lato complex found in the US and Europe, BB0172 has been highly conserved [51].

KLH:PepB conjugated antigen offered effective protection in the murine model of LD at low doses. However, when challenging animals using a high infective dose (10^5 spirochetes per mouse), this vaccine candidate failed to be protective. To improve its immunogenicity and capacity for long-term protection, in Chapter III, we conjugated PepB to carrier molecules, including TTHc and CRM197. Millions of people around the globe have been immunized with conjugate vaccines that make use of carrier proteins. The two most common carrier proteins used in glycoconjugate vaccines are Cross-Reacting-Material-197 (CRM197) and tetanus toxoid heavy chain (TTHc). Furthermore, information about the immunogenicity and safety profile of carrier proteins in clinical use is widely available [309].

Generally, ticks are responsible for approximately 95% of the vector-borne diseases reported annually in the US [10]. Also, they are second only to mosquitoes as vectors of disease-causative agents in humans, and other animal species [363]. In the United States, LD is commonly present in the northeastern and upper midwestern states. The disease is transmitted by tick bites of *Ixodes scapularis* and *Ixodes pacificus* species in the eastern and western states respectively [9, 364]. In fact, ticks of the *Ixodes* genus are the main vectors for Lyme borreliosis infection. In the past decades potentially due to climate change, tick populations have been extending geographically to other areas especially in the north-central and northeastern states, and that might enhance the spread of *Borrelia* infection to other susceptible hosts [365].

The *Ixodes* ticks are considered three host ticks with four life stages, starting from egg, larva, nymph, and adult, and the life cycle usually takes approximately two to

three years to complete according to the environmental factors [366]. The cycle of pathogen transmission begins when uninfected ticks acquired the infection after feeding on an infected host. Of these hosts, the white-footed mouse (*Peromyscus leucopus*) is considered the most competent reservoir host for *B. burgdorferi* [202, 367]. In ticks, *B. burgdorferi* infection is transtadially maintained through the tick life stages. Therefore, after molting the next tick stage are able to transmit LD to naïve hosts. Consequently, ticks are important epidemiological factor in the distribution of the LD [368].

During a blood meal, *B. burgdorferi* can be transmitted by *Ixodes* ticks to the vertebrate hosts. Ticks need to take blood meal to complete their development and life cycle, as obligate blood feeding ectoparasites. During feeding, ticks usually inject salivary proteins at the bite-site to assist in the tick feeding process and also transmission of pathogens. In fact, Ixodid tick saliva play a major role in reducing inflammatory reactions against vector-borne pathogens [369]. Different pathogens can utilize tick salivary molecules to promote their survival and multiplication within the vector, as well as transmission to the hosts [370-372]. Tick salivary molecules have anti-hemostatic, anti-inflammatory, and immunomodulatory functions that mediate transmission and establishment of pathogens in the hosts [32, 373, 374]. For instance, the salivary protein Salp15 facilitates *B. burgdorferi* transmission and replication in the hosts by inhibiting complement mediated killing, and also suppressing CD4 (+) T cell responses [35, 36]. Thus, it is imperative to test protection induced by novel vaccine candidates using the tick model of infection, since there could be different outcomes in the evaluation of vaccine efficacy studies when infection occurs via the tick vector [62].

Materials and methods

Ethics statement

All animal experiments and related procedures were followed the animal use protocol (AUP #2017-0022) that was approved by the Institutional Animal Care and Use Committee of Texas A&M University. Mice were kept and taken care of in an animal facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC).

Growth conditions of Borrelia burgdorferi

Borrelia burgdorferi B31 A3 strain was used throughout this study. It was grown in BSK II media supplemented with 6% inactivated naive rabbit serum (iNRS) and pH 7.6 under incubation conditions of 1% CO_2 and 32°C. Once the culture reached a cell density of 2×10^7 spirochetes/ml, the culture was used to infect naïve nymphs of *Ixodes scapularis* that were used to challenge the immunized animals.

Antigen administration and immunization protocol

Based on the results described in Chapter III, TTHc:PepB was the only vaccine candidate that was able to protect immunized mice from infection at high infective doses. Therefore, in this chapter we will be using TTHc:PepB as the only vaccine candidate to test, using the tick model of infection. To this end, and as described in Chapter III, groups of 6-8 week old C3H/HeN mice (n=8) were immunized with either TTHc, or TTHc:PepB at 0, 2 and 4 weeks. The mice were primed subcutaneously with (50 µg/mouse) of each antigen formulated in 10% Adjuplex® adjuvant. The vaccination

of mice was followed by two boosting doses at 14- and 28-days post-priming, in which mice received 10μg/mouse and 5μg/mouse of antigen respectively. A group of 4 mice served as the negative controls receiving 10% Adjuplex® adjuvant in saline. To evaluate protection, animals were challenged with *B. burgdorferi* using the tick model of infection at either 8-weeks post-priming (high specific IgG in serum) or 12-weeks post-priming (low specific IgG in serum).

Infection of Ixodes scapularis nymphs with B. burgdorferi

Naive nymphs of *I. scapularis* were purchased from Oklahoma State University Tick Laboratory, and were kept in perforated screw cap tubes in an environmental chamber at 25ºC, a relative humidity (RH) of 95% and a light/dark cycle of 9hrs/15hr. Prior to infection by immersion, nymphs were placed in a jar containing saturated ammonium sulfate $(NH_4)_2SO_4$ to maintain a RH of about 79% to slightly dehydrate the ticks, making them more prone to imbibe fluid during immersion. After incubation of nymphs for 72 hours at 79% RH, they were infected with *B. burgdorferi* by immersing them in a culture suspension containing 10^8 spirochetes/ml for 45 minutes at 32°C. Infected nymphs were then washed with sterile distilled water and incubated at 95% RH and 25°C for 72 hours in the environmental chamber. Prior to mouse infestation, infected ticks were transferred back to the jar at 79% RH. After 72 hours, the infected nymphs were ready to be brushed on mice. At either 8-weeks or 12-weeks post-priming, the immunized C3H/HeN mice were challenged by brushing 8 to 10 infected nymphs on each mouse. To this end mice were sedated, normal saline was provided subcutaneously, and ticks brushed on them. After brushing the ticks, mice were placed in individual

restrainers covered with white cloth so as to prevent the ticks from escaping the cages and to force them onto the mice. Four to six hours post-infestation, mice were housed in wire bottom cages for 10 days or until all nymphs feed to repletion and drop off the mice, upon which point fallen nymphs were collected and stored in 70% ethanol for further molecular analysis of tick spirochete load, as used previously [79]. The protective efficacy of the immune response was evaluated by sampling the immunized animals at 4-weeks post-challenge. At this point, mice were euthanized following IACUC recommended protocols, and blood and tissues were collected from each animal to evaluate antibody levels, bacterial clearance and bacterial burden. Collected tissues were: spleen, lymph nodes, tibiotarsal joint, heart, bladder and skin.

Enzyme linked immuno-sorbent assay

Immunized mice were euthanized at 4-weeks post-challenge, and sera were collected to evaluate IgG titers directed against the antigens PepB and the whole cell lysates of *B. Burgdorferi* A3 strain. 96 well plates (Nunc Maxisorb®, Thermo Scientific, Ltd.) were coated with (100 ng/well) BSA:PepB antigen, another set of plates were coated with the whole cell lysates of *B. Burgdorferi* A3 strain (10^7 spirochetes/well). The cell lysate was prepared from *Borrelia* culture that was shifted from RT/pH7.6 to 37°C/pH6.8. The antigens were coated overnight at 4°C. After washing excess coating antigen, serum samples were loaded in 2-fold serial dilutions starting from 1:100 to 1:102,400 and incubated for 1hr at room temperature. After washing, specific IgG levels were detected by adding 1:3000 dilution of the secondary anti-mouse HRP conjugated antibodies to the plates. Finally, the reaction was detected by adding *o*-

Phenylenediamine dihydrochloride (OPD) (Pierce, Thermo Scientific, Ltd) color substrate, and read at the optical density (OD) of 450 nm by using the Bio-Tek Synergy™ H1 microplate reader and Gen5™ software (BioTek Instruments Inc., Winooski, VT).

Evaluation of protective efficacy of vaccine candidates

Immunized mice were euthanized at 4-weeks post-challenge, and blood and tissues were collected to evaluate protection of studied antigens. Tissues of skin, spleen, lymph nodes, heart, bladder, and tibiotarsal joints were collected for bacterial recovery in BSK-II media enriched with 6% iNRS. Following 5 days of incubation at 32°C and 1% CO_2, 100 µl of the cultured media were blindly passed to fresh media, and incubated for 15 days before checking the bacterial growth under dark field microscopy [311]. In addition, parts of skin, spleen, lymph nodes, and tibiotarsal joints were processed for DNA extraction using the High Pure PCR Template Preparation Kit (Roche Applied Science, Indianapolis, IN), and they were used to evaluate bacterial burden by quantitative real-time PCR as previously illustrated [375].

Statistical analysis

All ELISA data were analyzed with Two-way ANOVA to evaluate the statistical difference within immunized groups. The quantitative real-time PCR data were analyzed by Kruskal-Wallis test to statistically compare between groups. The software Prism 7.0 (GraphPad Software, Inc.) was used to make all graphs and analysis in this study.

Results

Efficacy of TTHc:PepB vaccine candidate

Evaluation of humoral immune response in the immunized animals post-challenge was revealed by ELISA. In immunized mice that were challenged at 8 weeks post-priming, the anti-PepB antibody titers were relatively high compared to the control and TTHc groups, whereas anti-*Borrelia* antibody levels were comparably high (10,000) in all animal groups (Figure 4.1).

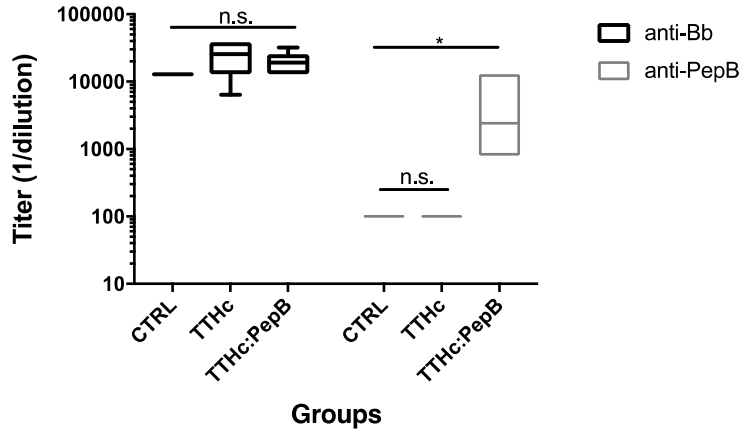

Figure 4.1. Elevated IgG antibody titers were detected in the immunized and infected mice against peptide B (grey bars) and *Borrelia burgdorferi* (black bars) at 8 weeks via tick challenge. Animals were infested with infected *Ixodes scapularis* ticks 8-weeks post-priming. TTHc:PepB induced significant high anti-PepB IgG titers compared to the control groups.

Furthermore, challenged animals at 12-weeks post-priming, TTHc:PepB immunized mice had significantly higher anti-PepB IgG titers (1:10,000) than the control and TTHc groups. On the other hand, anti-*Borrelia* IgG titers were high

(1:20,000) in all animal groups (Figure 4.2), denoting that they all got infected through the tick vector.

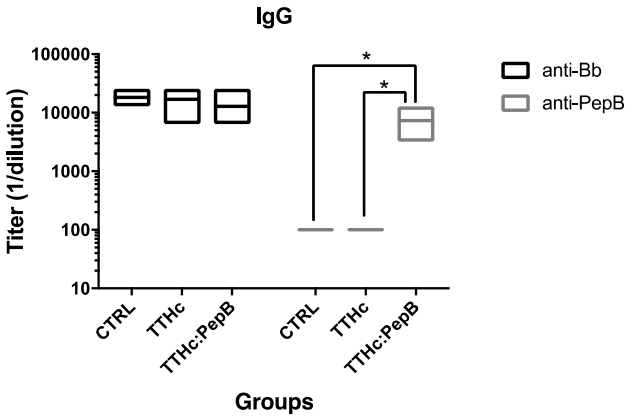

Figure 4.2. High IgG antibody titers were detected in the immunized and infected mice against peptide B (grey bars) and *Borrelia burgdorferi* (black bars) at 12 weeks by tick challenge. Animals were infested with infected *Ixodes scapularis* ticks 12-weeks post-priming. TTHc:PepB induced significant high anti-PepB IgG titers compared with the control. * Denotes statistical significances (* P value < 0.05; ** P value < 0.01) when compared with control groups.

Immunized C3H/HeN mice were infested at 8-weeks post-priming with infected nymphs. Mice were euthanized at 4-weeks post-challenge, and the bacterial burden was evaluated by qPCR in the harvested tissues which include skin, spleen, lymph nodes, and tibiotarsal joints. The qPCR results showed that TTHc:PepB significantly reduced bacterial burden in immunized mice compared to the control group (Figure 4.3). This reduction was especially obvious in skin, spleen, and tibiotarsal joints. Whereas, immunized C3H/HeN mice that were challenged with infected nymphs at 12-weeks post-priming showed reduction in bacterial burden of skin, spleen, lymph nodes, and tibiotarsal joints (Figure 4.4). It was shown that animals that were infected at 8 and 12-weeks post-priming, TTHc:PepB immunized mice in both groups showed a reduction in

bacterial burden of up to 70% in collected tissues compared to the control group (Figure 4.5).

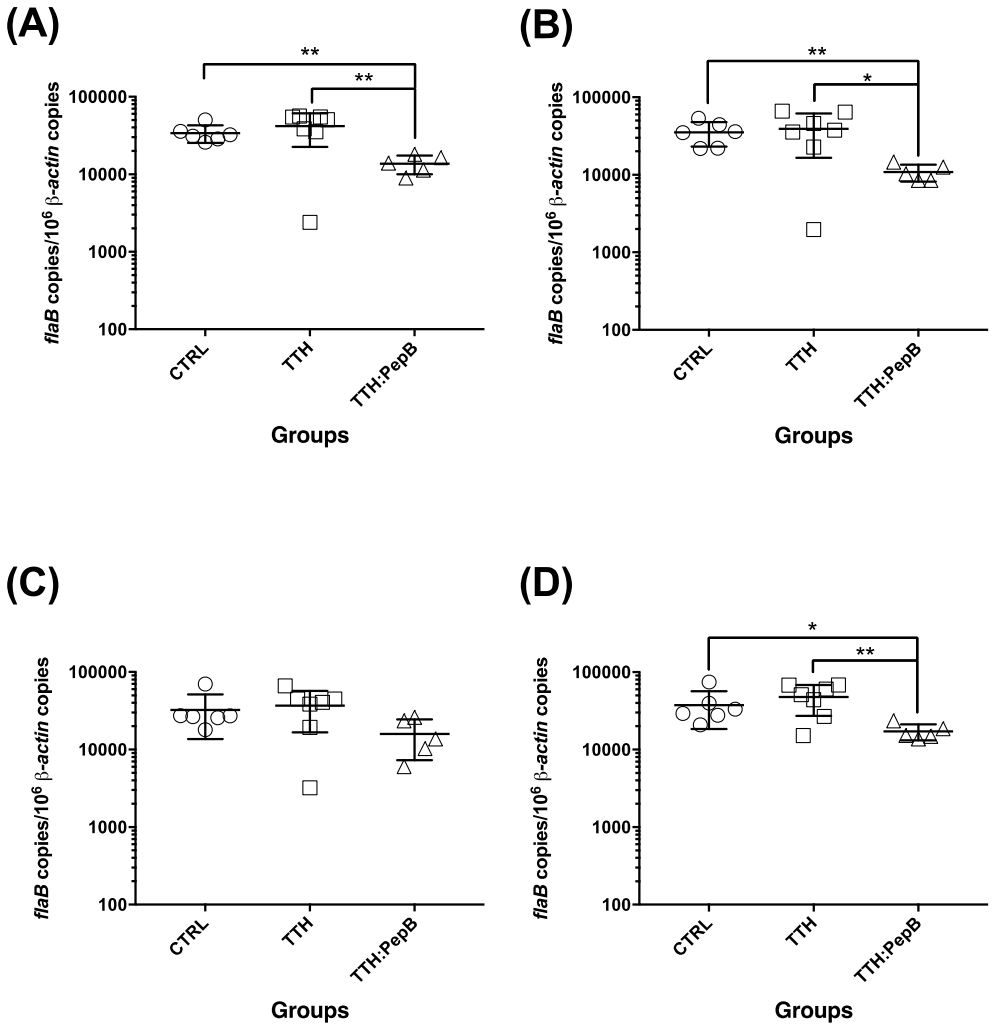

Figure 4.3. TTHc:PepB immunized mice display lower bacterial burden 4-weeks post-infestation with *B. burgdorferi* infected ticks 8-weeks post-priming. Bacterial burden was significantly reduced in mice immunized with TTHc:PepB in all examined tissues including skin **(A)**, spleen **(B)**, and joints **(D)** compared with the control. No significant reduction was observed in lymph nodes **(C)**. * Denotes statistical significances (* *P* value < 0.05; ** *P* value < 0.01) when compared with control groups.

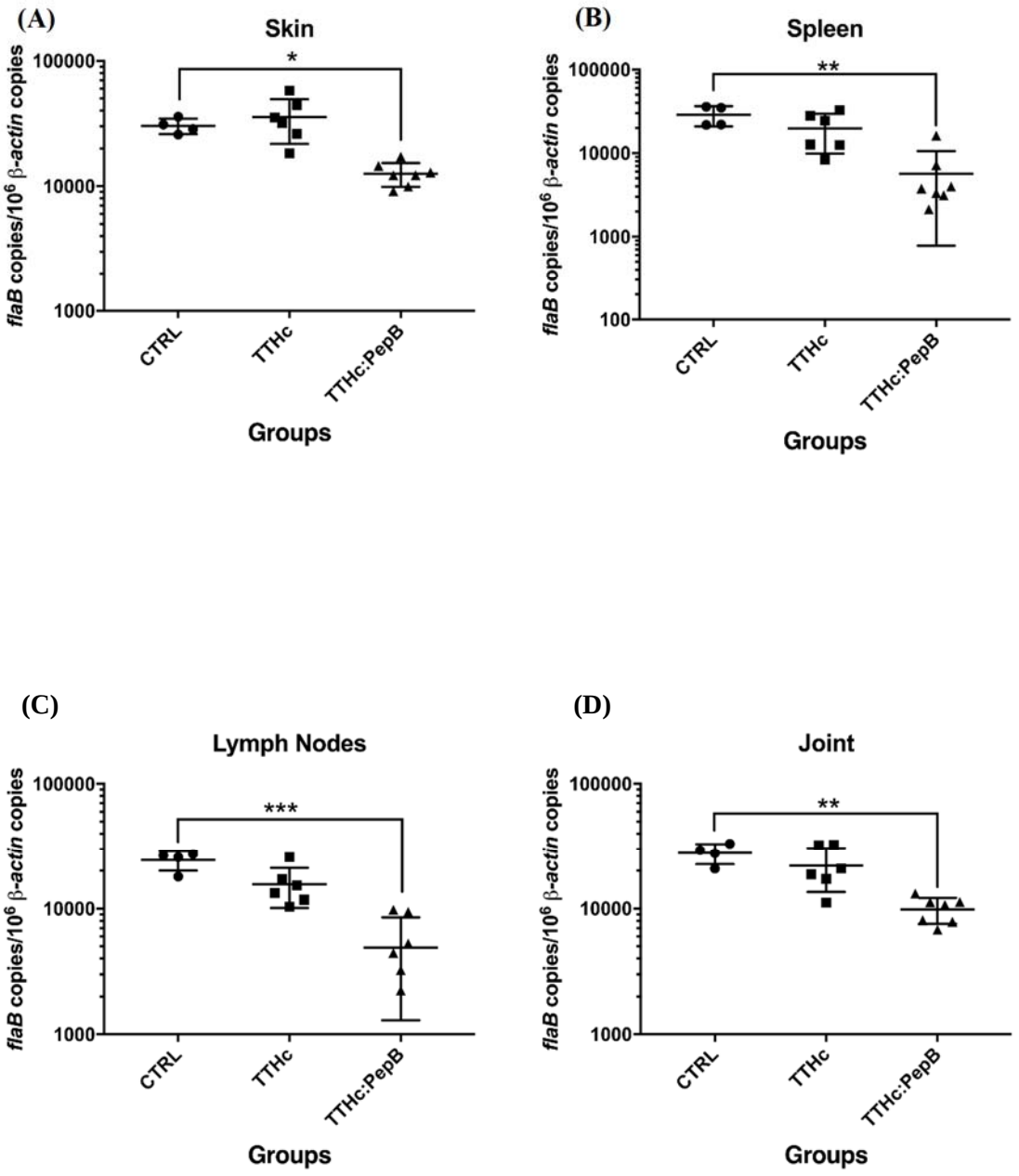

Figure 4.4. TTHc:PepB immunized mice display lower bacterial burden 4-weeks post-infestation *with B. burgdorferi* infected ticks 12-weeks post-priming. Bacterial burden was significantly reduced in mice immunized with TTHc:PepB in all examined tissues including skin **(A)**, spleen **(B)**, lymph nodes **(C)**, and joints **(D)** compared with the control. * Denotes statistical significances (* P value < 0.05; ** P value < 0.01) when compared with control groups.

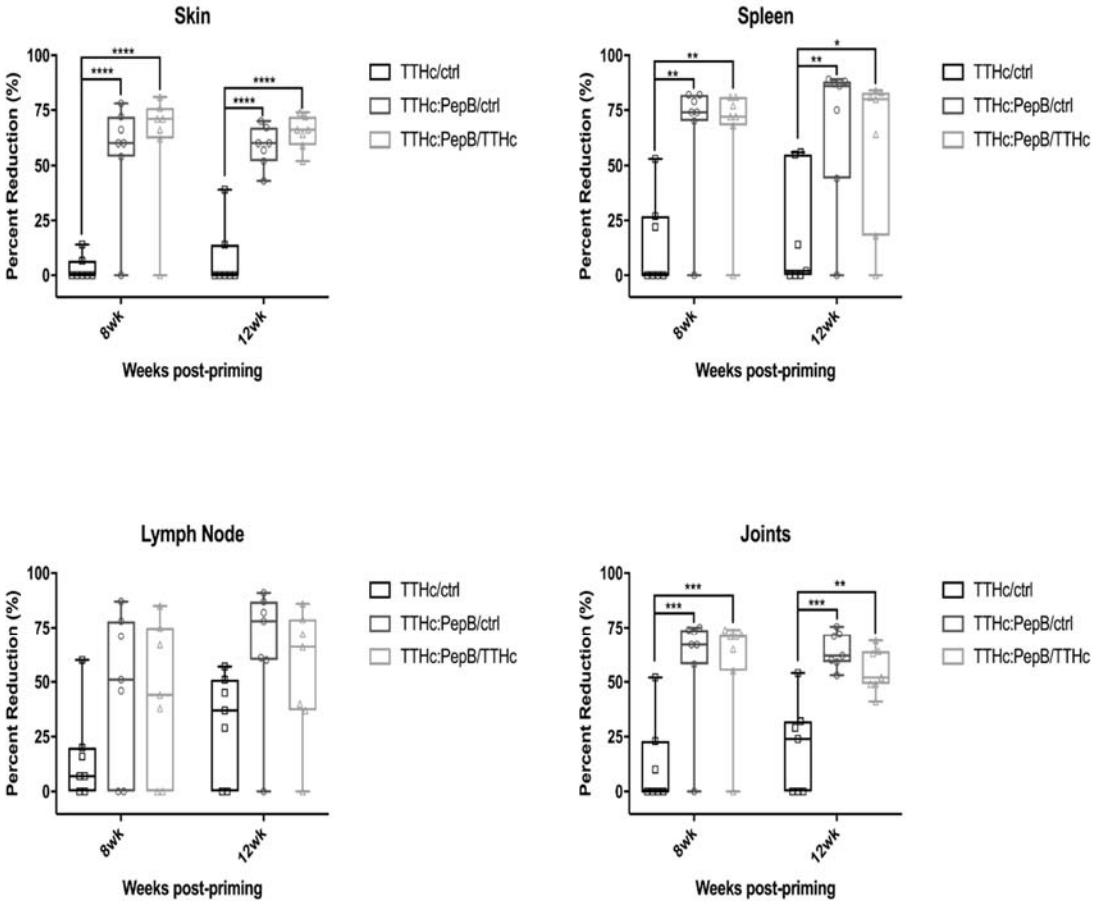

Figure 4.5. TTHc:PepB immunized mice reduced up to 70% bacterial burden in tissues at 8 and 12-weeks post infestation with *Ixodes scapularis* ticks infected with *B. burgdorferi*. * Denotes statistical significances (* *P* value < 0.05; ** *P* value < 0.01) when compared with control groups, n.s.: denotes not significantly differences.

Protection was evaluated 4 weeks post-infestation by determining bacterial recovery from different tissues by growth in BSK-II medium. When animals were infested 8 weeks post-priming, *B. burgdorferi* was recovered almost from all tissues except the bladders from 2 mice, and one spleen and tibiotarsal joint in the group receiving TTHc:PepB antigen (Table 4.1). In contrast, in animals that were challenged at 12-weeks post-priming, *Borrelia burgdorferi* was recovered from all collected tissues of all animal groups, except 1 tissue (bladder) out of 7 in TTHc:PepB group (Table 4.2).

Table 4.1. Recovery of *B. burgdorferi* from sampled tissues of infected and immunized mice at 8 weeks post-priming and sampled at 4 weeks post-tick infestation

Group	Skin	Lymph Node	Spleen	Bladder	Heart	Joint	Total
Control	4/4	4/4	4/4	4/4	4/4	4/4	24/24
TTHc	7/7	7/7	7/7	7/7	7/7	7/7	42/42
TTHc:PepB	5/5	5/5	4/5	3/5	5/5	4/5	26/30

No. of tissues positive/No. of tested tissues

Table 4.2. Recovery of *B. burgdorferi* from sampled tissues of infected and immunized mice at 12 weeks post-priming

Group	Skin	Lymph Node	Spleen	Bladder	Heart	Joint	Total
Control	4/4	4/4	4/4	4/4	4/4	4/4	24/24
TTHc	6/6	6/6	6/6	6/6	6/6	6/6	36/36
TTHc:PepB	7/7	7/7	7/7	6/7	7/7	7/7	41/42

No. of tissues positive/No. of tested tissues

Discussion

In this study, we used TTHc:PepB conjugate to immunize C3H/HeN mice, as it was the most efficacious antigen formulation as demonstrated in Chapter III. Our results showed that, after tick infection, TTHc:PepB immunized animals are unable to clear the infection as observed in Chapter III in which immunized animals were challenged using needle infections. Nevertheless, experiments performed in Chapter IV demonstrate a significant reduction of up to 70% in the bacterial burden of infected animals immunized with the TTHc:PepB antigen formulation. Therefore, the presented results are encouraging and deserve further investigation.

Ixodes ticks are primarily responsible for transmission of LD through their hematophagous behavior of feeding. During the blood meal, tick saliva components play a major role in the transmission and survival of the tick-borne pathogens. Of these, salivary gland proteins such as salp15 was shown to have host immunosuppressive traits including inhibition of $CD4^+$ T-cells activation [36]. Also, it facilitates survival of spirochetes within the infected host through binding to OspC lipoprotein [35]. There are other salivary secretions involve in the survival and enhancement of the infectivity of spirochetes within the infected hosts. For instance, Salp20 has been shown to promote tick feeding and protect tick-borne pathogens from the complement system [376]. Moreover, the tick salivary lectin pathway inhibitor (TSLPI) has been identified in *Ixodes* ticks, and it has a major role in the inhibition of the vertebrate complement cascade. Through which it was shown that it enhanced the acquisition and persistence of *Borrelia* in tick midguts, and *Borrelia* transmission as well [33]. The tick histamine

release factor (tHRF) is also another salivary protein that acts by stimulation of histamine release in the host, and thereby assist in the tick feeding and transmission of tick-borne agents [61].

The laboratory adapted culture of *Borrelia burgdorferi* might not be suitable for conducting infectious challenges, since it does not exhibit the same protein expression patterns as the spirochetes within the ticks that adapt their surface structures to the tick environment [377, 378]. However, all the essential plasmids in the *B. burgdorferi* were confirmed by PCR screening method before we used it in the needle challenge study. In addition, there are several differences between cultured *B. burgdorferi* and spirochetes growing *in vivo* [379, 380] that may affect our results in this study. Based on that, it may be concluded that the amount and accessibility of the upregulated BB0172 derived antigen on the exposed surfaces of spirochetes *in vivo* may be altered compared to those of cultured bacteria. Moreover, other factors that may restrict the interaction of the PepB-specific antibodies with *B. burgdorferi* within the host, are the ability of these spirochetes to widely disseminate throughout their host, and also the paucibacillary nature of *B. burgdorferi* infection [381]. Infected ticks have been used for challenge protocol in this study. In the infectious tick bite, the inoculated dose of spirochetes is not controlled compared to the known dose administered via needle inoculation.

However, it was previously shown that on week 12 post-priming the anti-Pep B antibody levels had reduced to basal levels [79]. The memory immune response was effectively triggered in the immunized mice since those animals were shown to mount an immune response following tick challenge. This confirms that TTHc:PepB successfully

induced T-cell dependent immune response, which may also account in the reduction of bacterial burden we observed in the immunized animals. This is consistent with a previous study that showed that $CD4^+$ T cells were required for the control of spirochetes load and amelioration of arthritis [382].

In bacterial recovery, immunized mice were challenged with infected ticks at 8-weeks post-priming. It was showed that mostly all cultured tissues from the challenged mice were able to revive *B. burgdorferi*. However, anti-PepB antibodies reached their peak levels at 8-weeks post-priming, but they were ineffective to confer protection against the *B. burgdorferi* infection. Also, immunization with TTHc:PepB was not quite efficient to prevent growth of spirochetes from the cultured tissues following the tick challenge at 12-weeks post-priming. Interestingly, it was shown that immunized animals tend to shed less *Borrelia* in the bladder as previously described [79]. Nevertheless, mostly all cultured tissues were shown to grow *B. burgdorferi*, and this is a different outcome in contrast to the needle inoculation experiment, in which we observed 66% protective rate in terms of bacterial recovery. Nevertheless, after tick infestation we still see reduced bacterial burden in the immunized animals compared to the control ones.

Ixodes ticks are the primary vectors for LD spirochetes. In the ticks, the population of spirochetes rapidly and highly expands during ticks feeding [383, 384]. In addition, it has been shown that administration of tick salivary gland extract was able to increase the dissemination and number of spirochetes in mice [385, 386]. Therefore, the viable bacterial inoculum is highly increased at the tick bite site, and it may exceed the dose of spirochetes that are inoculated using the needle challenge protocol. Furthermore,

as mentioned in the introduction, tick saliva proteins will protect Borrelial cells, increasing their infectivity. Along those lines, investigators have demonstrated that spirochetes show high virulence after they have been primed by a blood meal during tick feeding [387].

Overall, TTHc:PepB did not protect after tick infection, nevertheless, immunized animals tend to have significantly lower bacterial burden in different tissues, both at 8-weeks and 12-weeks post-priming. These results suggests that, other approaches could be explored to enhance the immunogenicity of PepB such as it uses in a multi-copy peptide or virus-like particles (VLPs), or the use of chimeric or multivalent vaccines where anti-tick and anti-LD antigens are administered in the same formulation. Furthermore, the improvement in the delivery methods of antigen could also be critical to induce long-term protection such as use of liposomes [388].

CHAPTER V

SUMMARY AND CONCLUSIONS

Lyme disease (LD) is the most prevalent vector-borne disease in the US, and it is transmitted by hard ticks of *Ixodes* spp. However, vaccine studies are still ongoing to develop a safe, efficacious, and broadly protective vaccine candidate against LD infection. Nevertheless, there is no currently available commercial vaccine against LD for use in human. Therefore, personal preventive measures are necessary to control transmission of LD infection, including selection of clothing, tick observation, and use of repellents.

Previously, the evaluation of BB0172 derived-peptides in the murine model of LD revealed that the PepB is a promising vaccine candidate that can be further investigated. Later, in this study PepB formulation was inserted in a protein scaffold BBA34, a lipoprotein of *B. burgdorferi* located in the outer membrane. BBA34:PepB construct was inefficient to stimulate a protective response in immunized mice against challenge with *B. burgdorferi*. Thus, the PepB might not be efficiently enhanced within BBA34 molecule *in vivo*. Therefore, other scaffolding molecules can be studied, and further alternative methods could be considered to enhance the immunogenicity of the PepB such as using carrier proteins, encapsulated vaccine formulation, and/or reformulation of the PepB in a polypeptide context.

Therefore, in our lab the exploring of novel approaches by studying BB0172 derived peptide as a vaccine candidate was to avoid the drawbacks of the classical outer

surface proteins in LD vaccines. The promising results of the conjugated PepB lead us to move to the next step of conjugation of the peptide with carrier molecules approved for human use.

In my work, the approach was to study the immunogenicity of the conjugated PepB with carrier molecules, including TTHc and CRM197. In that regard, TTHc:PepB conjugate was able to elicit protective immune response in immunized mice after needle challenge with *B. burgdorferi,* in which 66% protection was observed based on the bacterial recovery data. However, CRM197:PepB also, induced the generation of high antibody titers, but it was not as effective as TTHc:PepB.

TTHc:PepB construct was further evaluated using the tick infection model, as it was the most efficacious antigen in the needle challenge protocol. In that regard, in order to mimic the natural route of infection, experimentally infected ticks with *B. burgdorferi* were used to challenge the immunized mice. In this part of the study, one group of immunized mice were challenged at 8 weeks post-priming to evaluate the efficacy of the triggered humoral immune response at its peak level. Whereas, the other group of immunized mice were challenged at 12 weeks post-priming to evaluate the efficacy of TTHc:PepB in the stimulation of memory B-cells and confer protection against infection with *B. burgdorferi*. In both experiments, TTHc:PepB construct was unable to effectively clear the infection in the immunized mice. However, borrelicidal antibodies significantly reduced the bacterial burden in infected tissues of the immunized mice compared with the control group.

In future studies, it is important to consider further improvement in the PepB formulation to effectively enhance its protective efficacy. Different approaches could be explored in that regard including generation of synthetic, repetitive copies of the PepB molecule and /or enhance antigen delivery through encapsulated vaccine approach. Furthermore, the co-administration of PepB formulation with a potential anti-tick vaccine would be more effective to reduce the vector and pathogen burden in a host.

Furthermore, cross-protection efficacy of PepB can be evaluated by challenge against different genospecies of *B. burgdorferi sensu lato* complex. Based on that, PepB based vaccine formulation can be used not only in North America but also in other parts of the world as the other genospecies are prevalent in Europe and other parts of Asia.

In conclusion, study of the PepB formulations revealed that it induced high antibody titers in immunized animals, and also the anti-PepB antibodies did not cross react with *B. burgdorferi* lysate. In tick challenge, TTHc:PepB failed to effectively clear the infection in the murine model of LD, but significantly reduced bacterial burden in immunized individuals. Thus it is our conclusion that PepB formulation requires further optimization to enhance its immunogenicity and protection in the murine model of Lyme disease.

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