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Enhanced protective efficacy of *Borrelia burgdorferi* BB0172 derived-peptide based vaccine to control Lyme disease

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ABSTRACT

Lyme disease (LD) accounts for over 70% of tick-borne disease reported in the United States. The disease in humans is characterized by skin rash, arthritis, cardiac and neurological signs. Vaccination is the most efficient preventive measure that could be taken to reduce the incidence of the LD worldwide; however, at present no vaccine is available. In this study, evaluation of the *Borrelia burgdorferi* BB0172-derived peptide (PepB) in conjugated formulations was investigated as a vaccine candidate in murine model of LD. In brief, PepB was conjugated to the Cross-Reacting Material 197 (CRM197) and to Tetanus Toxoid heavy chain (TTHc) molecules, and subsequently used to immunize C3H/HeN mice. Following the challenge with 10^5 spirochetes/mouse via subcutaneous inoculation, TTHc:PepB construct showed protection in 66% of the immunized animals. Hence, to further evaluate the efficacy of TTHc:PepB, immunized mice were challenged with *B. burgdorferi* using the tick model of infection. The outcome of this experiment revealed that serum from TTHc:PepB immunized mice was borrelicidal. After tick infection, bacterial burden was significantly reduced (over 70%) in vaccinated animals when compared with the control groups regardless of whether the mice were infested 8 or 12-weeks post-priming. Therefore, we conclude that PepB conjugated antigens can serve as an alternative to prevent LD; nevertheless, further studies will be needed to dissect the mechanisms by which anti-PepB IgG antibodies are able to kill *B. burgdorferi* *in vitro* and *in vivo* to further advance in the development of formulations and delivery alternative to generate a safe anti-LD vaccine.

1. Introduction

Lyme disease (LD) is an infectious disease caused by a spirochetal bacterium *Borrelia* spp. transmitted by *Ixodes* ticks. In humans, the disease is characterized by fever, arthralgias, myalgias, and erythema migrans [1]. The Centers for Disease Control and Prevention (CDC) report more than 30,000 cases of LD annually, making it the most prevalent tick-borne infection in the United States. The majority of human cases of LD infection occur in Northeastern US during early spring and summer months due to abundance and activity of nymphal ticks [2].

The differentially regulated outer surface proteins of *B. burgdorferi* [3] have been targeted as vaccine candidates against LD. Of these, outer surface protein A (OspA) facilitates persistence and colonization of spirochetes within a tick's mid-gut via its receptor (TROSPA) [4]. Moreover, it has antigenic properties since antibodies directed against OspA were found to react with spirochetes isolated from LD cases [5–7].

Outer surface protein C (OspC) is expressed during the mammalian infection process and is also involved in transmission of *B. burgdorferi* from the tick vector to the mammalian host [8]. It has been shown that OspC immunized mice were protected when challenged with *B. burgdorferi* infected ticks [9]. However, there is significant heterogeneity in OspC among *Borrelia* spp. isolates [10]. Therefore, in order for an OspC-based vaccine to be effective, it must incorporate multiple heterologous, potentially protective epitopes expressed by the different *Borrelia* spp. isolates [11]. In fact, these observations have led to the evaluation of an octavalent, chimeric OspC vaccine candidate [12], as well as the development of a recombinant, heptavalent LD vaccine by Zoetis called VANGUARD® crLyme, originally formulated to prevent LD in dogs [13].

Currently, there is no vaccine against LD available for humans. Reverse vaccinology has enabled researchers to synthesize and test

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different potential vaccine candidates to induce protective immunity [14]. It provides information regarding the identification of surface-exposed antigens that can be tested either *in vitro* or *in vivo* models to effectively screen these candidates [15]. It is also a useful tool to recognize novel vaccine candidates through the genomic analyses of different isolates within the same species or between closely-related species [16,17]. Through this technology, our team has identified a short peptide (PepB) that induced a protective immunity against infection with *B. burgdorferi* [18]. 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 [19]. 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 37 °C (fed-tick conditions). However, *B. burgdorferi* in cultures adapted to either condition do not express BB0172 [19]. 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) [18]. Furthermore, BB0172 has been highly conserved among the different genotypes in the *B. burgdorferi* sensu lato complex found in the US and Europe [19]. PepB was shown to confer effective protection in the murine model of LD at low doses (10^3 spirochetes/-mouse) [18]; however, at a high dose (10^5 spirochetes/mouse) it failed to be protective. Furthermore, in that study PepB only induced partial protection using the tick model of infection.

Taking together, we propose to conjugate PepB to alternative carrier molecules, with the objective to further improve its immunogenicity and long-term protection. To this end, we immunized C3H/HeN mice with these antigens following a prime-boost immunization protocol and evaluated the safety and protective efficacy of conjugated PepB following needle and tick inoculation of virulent *B. burgdorferi* in both the control and immunized animals.

The carrier proteins selected for this study have been successfully used in conjugate vaccine to immunize millions of people around the globe. Of these, Cross Reactive Materia (CRM197) and Tetanus Toxoid Heavy chain (TTHc) are common carrier proteins used in glycoconjugate vaccines [20], and in conjugation with peptide antigens such as Improvest® (Pfizer Animal Health) [21]. The extensive body of information about their immunogenicity and safety profile in clinical setting is well-known [22].

2. Materials and methods

2.1. Ethics statement

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

2.2. Peptide design and conjugation

A 12 amino acid peptide (PepB) derived from the Borrelial outer membrane protein BB0172 was synthetized by Peptide 2.0 (Chantilly, VA) at a 95–98% purity. PepB sequence is not disclosed due to intellectual property disclosures currently deposited at Texas A&M University. After synthesis, PepB 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, PepB conjugated to Bovine Serum Albumin (BSA) was generated to evaluate antibody levels against PepB in traditional Enzyme Linked Immunosorbent Assays (ELISA).

2.3. 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 naïve rabbit serum (iNRS), to mimic the unfed-tick conditions. The culture was incubated until it reached a cell density of 1–2 $\times$ 10^7 spirochetes/ml, and then subcultured in BSK-II medium pH 6.8 plus 6% iNRS at 37 °C 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 $\times$ 10^7 spirochetes/ml. To prepare whole cell lysates antigen for ELISA, cells were harvested, washed three times with HBSS buffer (Thermo Scientific Inc.), counted, and lysed using 0.1 mm glass beads on a BeadRuptor 24 (Omni International, Inc.) at 5000 rpm for 5 min. Lysates were stored at −20 °C until use. For needle infection, *B. burgdorferi* cultures were prepared as above. Then, bacteria were harvested, washed twice with HBSS buffer, and resuspended in HBSS containing iNRS (50:50, *v:v*). Cell suspensions were quantified and adjusted to the required infective dose. For tick challenge, *B. burgdorferi* culture was grown in BSK II media supplemented with 6% iNRS and pH 7.6 in 1% CO_2 and 32 °C. Once the culture reached a cell density of 2 $\times$ 10 spirochetes/ml, cells were used to infect naïve nymphs of *Ixodes scapularis* ticks.

2.4. Immunization protocol

In this study, C3H/Hen mice were used as model for Lyme disease. To this end, six to eight-week old female C3H/HeN mice were divided into four groups with 9 mice each. To reduce the number of animals used avoiding duplication of experiments, two groups of mice served as control groups receiving the unconjugated carrier molecules, CRM197 for group 1 and TTHc for group 2. The other two groups received either conjugated peptide, CRM197:PepB (group 3) and TTHc:PepB (group 4). All groups were primed at 50 μg of the antigen subcutaneously (SQ) per animal with their respective antigen. Animals were boosted with 10 and 5 μg/animal SQ at days 14 and 28 post-priming respectively. 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 seroconversion. Three mice per each group were euthanized and samples from skin (near immunization site), heart, liver, kidney, and tibiotarsal joint were collected for histologic evaluation post-immunization.

To evaluate protection induced by both CRM197 and TTHc conjugated PepB formulations, immunized mice were challenge at 12-weeks post-priming with pathogenic *B. burgdorferi* via SQ inoculation. Furthermore, to confirm protection using the tick model, animals (n = 14) were immunized following the protocol described above, and were subsequently challenged via tick infestation at 8-weeks (n = 7) and 12-weeks (n = 7) post-priming. In these experiments, a control group (n = 7) receiving the carrier molecule and a second control group receiving the adjuvant only (n = 4) were included. This experiment was done with the vaccine conjugate that conferred protection wia needle infection.

2.5. Borrelia burgdorferi needle challenge

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 $\times$ the infectious dose 50 (ID_{50})) subcutaneously in HBSS containing iNRS (*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, urinary bladder, and tibiotarsal joint. Tissues were cultured in BSK-II media as descrived above. The cultures were blindly

passed into fresh BSK-II media after 5 days post-initial inoculation and incubated at 32 °C 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 [23].

We evaluated bacterial burden in skin, spleen, inguinal lymph node, and tibiotarsal joint by quantitative real time (qPCR) as previously described [24]. Finally, fragments of skin, heart, liver, kidney, and tibiotarsal joint were collected for histopathology.

2.6. Tick infection challenge

The antigen inducing protection in the needle challenge experiment was also tested using the tick model of transmission. To this end, animals were challenged with *Ixodes scapularis* nymphal ticks infected with *B. burgdorferi* B31 stain A3. This challenge was performed at 8-weeks post-priming where antibody titers are at their maximum, and at 12-weeks post- priming, where antibody titers were declining to basic levels. As described above, 14 six to eight week old C3H/HeN mice were immunized with TTHc:pepB, another 14 mice with TTHc and 6 naïve mice were used as controls. Half of the animals in each group were infested at 8-week post-priming, and the other half at 12-weeks post-priming. To prepare the ticks for infection, *B. burgdorferi* was grown in BSK-II media pH 7.6, and screened for the presence of plasmids required for infectivity [25]. In tick infection method, naïve *Ixodes scapularis* nymphs were purchased from Oklahoma State University Tick Laboratory and were incubated in a desiccator jar at RT and 79% relative humidity (RH) for 4 days. Then, ticks were immersed in a *B. burgdorferi* suspension at 10^8 *B. burgdorferi*/ml for 45 min, and washed twice with PBS to remove the excess media. Ticks were incubated at 90% RH for 4 days to enhance recovery. After recuperation, infected ticks were shifted to a desiccator jar at 79% RH for 3 days prior to mouse challenge. To challenge the immunized mice, 8 to 10 infected nymphs were brushed onto each mouse, and each animal was individually housed in wire-bottom cages for 10–14 days or until infected nymphs feed to repletion. Engorged nymphs were collected and kept in 70% ethanol. Four weeks post-infestation, mice were euthanized and samples were collected as described above.

2.7. Histopathology

Tissues were collected from immunized mice on week 8 post-priming, and 3-weeks after needle challenge. Tissues were preserved in 10% formalin, while tibiotarsal joints were decalcified in 10% EDTA for 4 weeks prior to further processing. All tissues were routinely processed for histology and stained with hematoxylin and eosin. 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 to 4. Inflammatory scores ranged from normal = 0 (no inflammation), minimal = 1 (one focus of inflammation), mild = 2 (2–5 foci of inflammation), moderate = 3 (>5 foci of inflammation), and severe = 4 (multifocal to diffuse, with more than 30% of section infiltrated with inflammatory cells) [26].

2.8. Enzyme-linked immunosorbent assay (ELISA)

The 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 4 °C with PepB conjugated to BSA at a concentration of 100 ng/well (Fina Biosolutions LLC, Rockville, MD). To evaluate antibody response to borrelial whole cell lysate, *B. burgdorferi* A3 strain grown 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 containing 0.1% Tween 20, (PBS–T)) and blocked with blocking buffer (PBS–T containing 3% BSA) for 1 h at RT. Following three washes with PBS–T, mice sera were loaded to the wells in duplicates following 2-fold serial dilutions in dilution buffer (PBS–T containing 1% BSA) ranging from 1:100 to 1:102,400 across the plate. Plates were incubated for 1 h at RT, followed by three washes with PBS–T. Plates were subsequently incubated with secondary conjugated anti–mouse antibody (Horseradish Peroxidase, HRP) diluted 1:3000. 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).

2.9. 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 and quantity of DNA, the presence of *B. burgdorferi* was evaluated by quantitative real time PCR (qPCR) as previously described [23]. Final results were reported as borrelial *flaB* gene copies per 10^6 copies of mouse *β-actin* (*flaB* copies/10^6 *β-actin* copies). All primers and amplification conditions have been described elsewhere [24,27].

2.10. 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 as described above. 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 diluted 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 1 h at 37 °C, followed by the addition of 1:10 dilution of commercially available rabbit complement (Millipore Sigma). The mixtures were incubated for an additional 1hr at 37 °C. Borrelial survival was measured before and after the addition of rabbit complement by doing 10-fold serial dilutions in fresh BSK-II media complemented with 6% iNRS, and incubating the cells at 32 °C and 1% CO_2. Bacterial growth was evaluated under dark field microscopy at 48 and 72 h.

2.11. Statistical analysis

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

3. Results

3.1. Needle inoculation challenge:

3.1.1. Efficacy of PepB conjugated vaccine candidate

In order to evaluate humoral immune response of immunized mice, sera from 3 animals of each group were collected at 8-weeks post-priming. As shown in Fig. 1, high IgG titers

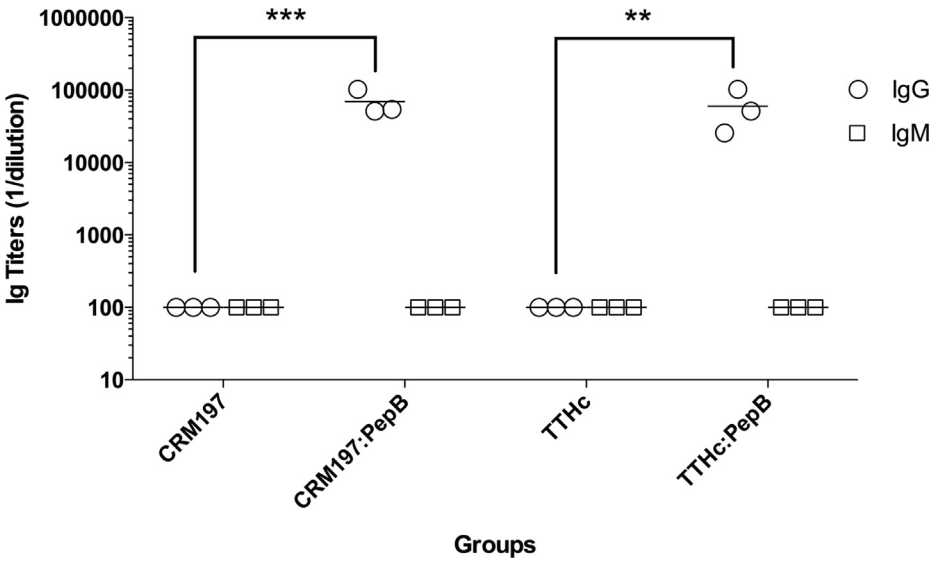

Fig. 1. Antibody response post-immunization with PepB conjugated vaccine candidates. High IgG titers were detected at 8–weeks post–priming, while IgM titers of immunized animals were low at basal levels. * Denotes statistical significances (** P value < 0.01; *** P value < 0.001) when compared with control groups.

(1:100,000) were observed in all 3 animals immunized with conjugated PepB vaccines, compared to low titer of 1:100 in both carrier molecule immunized animals (CRM197 and TTHc groups). In regards to IgM levels, they were present at low titer (1:100) in all animals from all conjugated PepB groups, as well as in the unconjugated CRM197 and TTHc groups.

The immune response of animals after challenge via subcutaneous needle inoculation with 10^5 spirochetes/mouse was detected by tracking antibody titers in the serum samples which were collected at 3–weeks post–challenge. As shown in Fig. 2A, immunized animals with either conjugated PepB showed significantly higher IgG titers (1:10,000) compared to the results observed in animals receiving CRM197 and TTHc, which were at basal levels (1:100). On the other hand, ELISA results showed that anti-*B. burgdorferi* specific IgG titers (Fig. 2B) were generally high (1:10,000) in all immunized and infected animal groups, except one animal from TTHc:PepB group (1:100). Consistent results were observed when the experiments were repeated (Supplemental Fig. 1).

Evaluation of IgG isotypes to determine whether Th1 and/or Th2 immune response was induced in immunized animals post-infection with *B. burgdorferi* showed significant increase in IgG_1 titers compared to the unconjugated groups. Also, IgG_{2a} titers showed the same trend as IgG_1 (Fig. 2C).

Evaluation of protective efficacy of the conjugated PepB 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 SQ inoculation of 10^5 spirochetes/mouse. Mice were euthanized at 3-weeks post-challenge, and bacterial burden in animal tissues including skin, spleen, lymph node, and tibiotarsal joints was determined by qPCR. 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* (Fig. 3). However, in animals receiving the CRM197:PepB antigen, the spirochetal load in skin, lymph nodes, and joints was relatively low compared to the CRM197 group.

Borrelia burgdorferi was recovered in culture from 2 out of 7 mice immunized with TTHc:PepB. However, *B. burgdorferi* was recovered from all tissues of all other immunized and infected animals except those in the CRM197:PepB group, where *B. burgdorferi* was recovered from 4 out of 6 skin cultures (Table 1). In regard to TTHc:PepB group, this experiment was repeated to confirm our results, with recovery of *B. burgdorferi* from only 2 out of 5 mice (Table 1). Overall, considering both biological replicates, it was estimated that TTHc:PepB conferred 66% protection in immunized mice when infected with a $1000 \times ID_{50}$.

3.1.2. Evaluate safety of administered antigens

The safety of TTHc:PepB and CRM197:PepB in immunized animals was evaluated by histopathology at 8-weeks post-priming and 4-weeks post-needle infection. These antigens are considered safe when they do not inducing severe inflammation or significant cellular injury post-inoculation. When present, the inflammatory cells were composed of lymphocytes, plasma cells, and occasional neutrophils. CRM197:PepB immunized mice showed a minimal to mild inflammatory response in the heart, kidney, and tibiotarsal joint after infection. On the other hand, TTHc:PepB and TTHc immunized animals showed minimal to mild inflammation only in the liver after immunization. Overall, our experiments did not reveal significant inflammation in either group after immunization or infection (Fig. 4), furthermore, the inflammation observed was most severe in the CRM197:PepB groups.

3.1.3. Evaluate the bactericidal activity of immunized mouse serum

The serum bactericidal activity was measured in sera of immunized and control mice using commercially available rabbit complement. The antibodies from immunized mouse sera regardless of the vaccine conjugate used (CRM197:PepB and TTHc:PepB) were effective in reducing the number of *B. burgdorferi* cells at all tested dilutions (1:50, 1:100, 1:1000, and 1:2000) when compared to the negative control group (Fig. 5).

3.2. Tick challenge study

3.2.1. Efficacy of TTHc:PepB vaccine candidate

In immunized mice that were challenged at 8-weeks and 12-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 (>1:10,000) in all animal groups (Fig. 6A and B). These results denote that all animals got infected through the tick vector and that no differences were

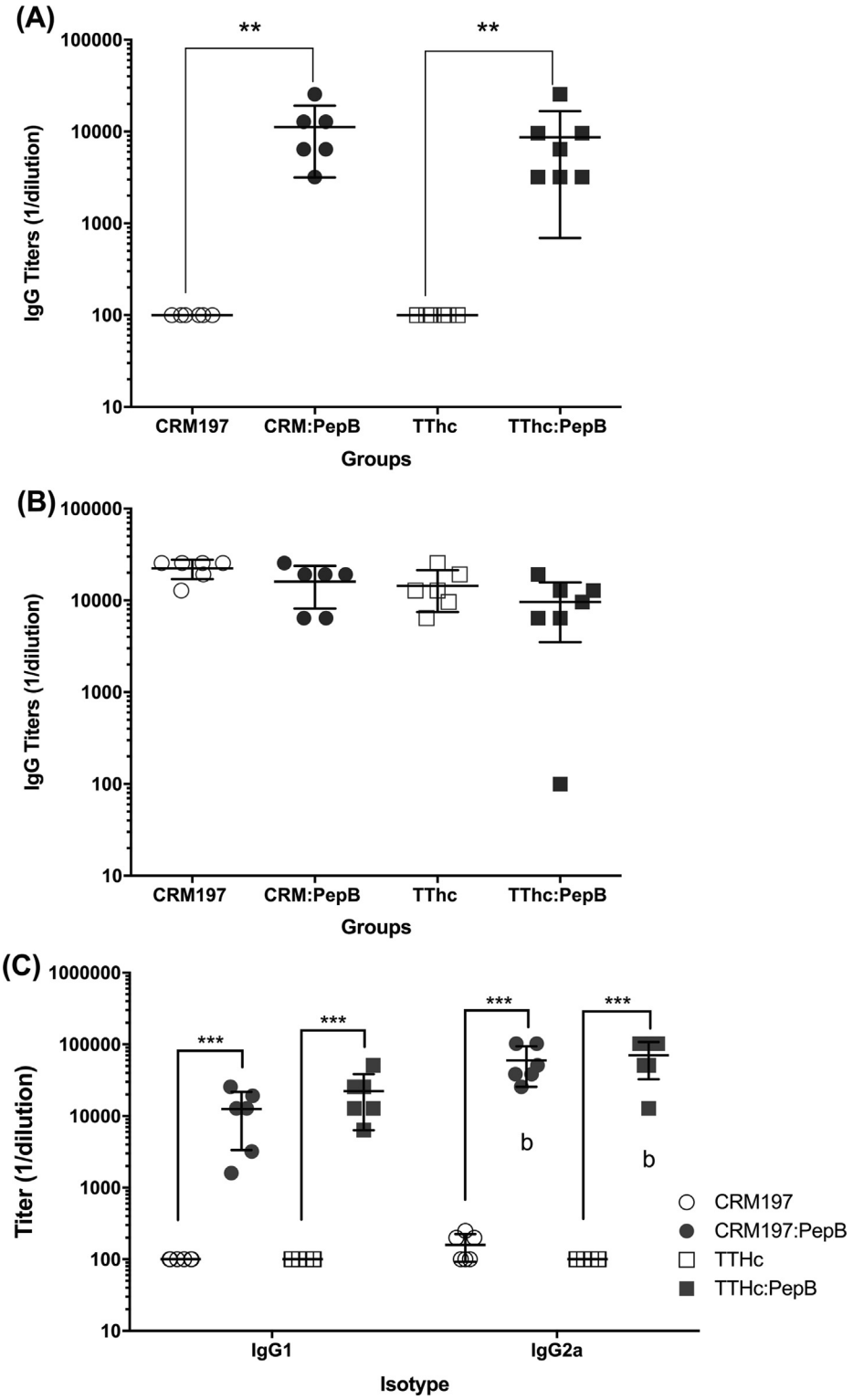

Fig. 2. High IgG antibody titers were detected in immunized and infected mice against peptide B (A) and *Borrelia burgdorferi* (B) at 3 weeks post needle infection. TTHc:PepB and CRM197:PepB induce significantly higher IgG titers compared with TTHc and CRM197. (C) IgG1 and IgG2a antibody titers were evaluated at 3 weeks post needle infection, showing significant increase of both isotypes in animals receiving the PepB conjugated vaccines. * Denotes statistical significances (** 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).

observed between each experiment, demonstrating a consistency in the tick artificial infection procedure developed in the laboratory.

The bacterial burden was evaluated in the immunized mice that were infested at 8-weeks post-priming with infected nymphs. The qPCR results showed that TTHc:PepB significantly reduced bacterial burden in immunized mice compared to the control group (Fig. 7). This reduction was significant in the skin, spleen, and tibiotarsal joints, similarly to what was observed when animals were infested with infected ticks 12-weeks post-priming (Fig. 7). It is

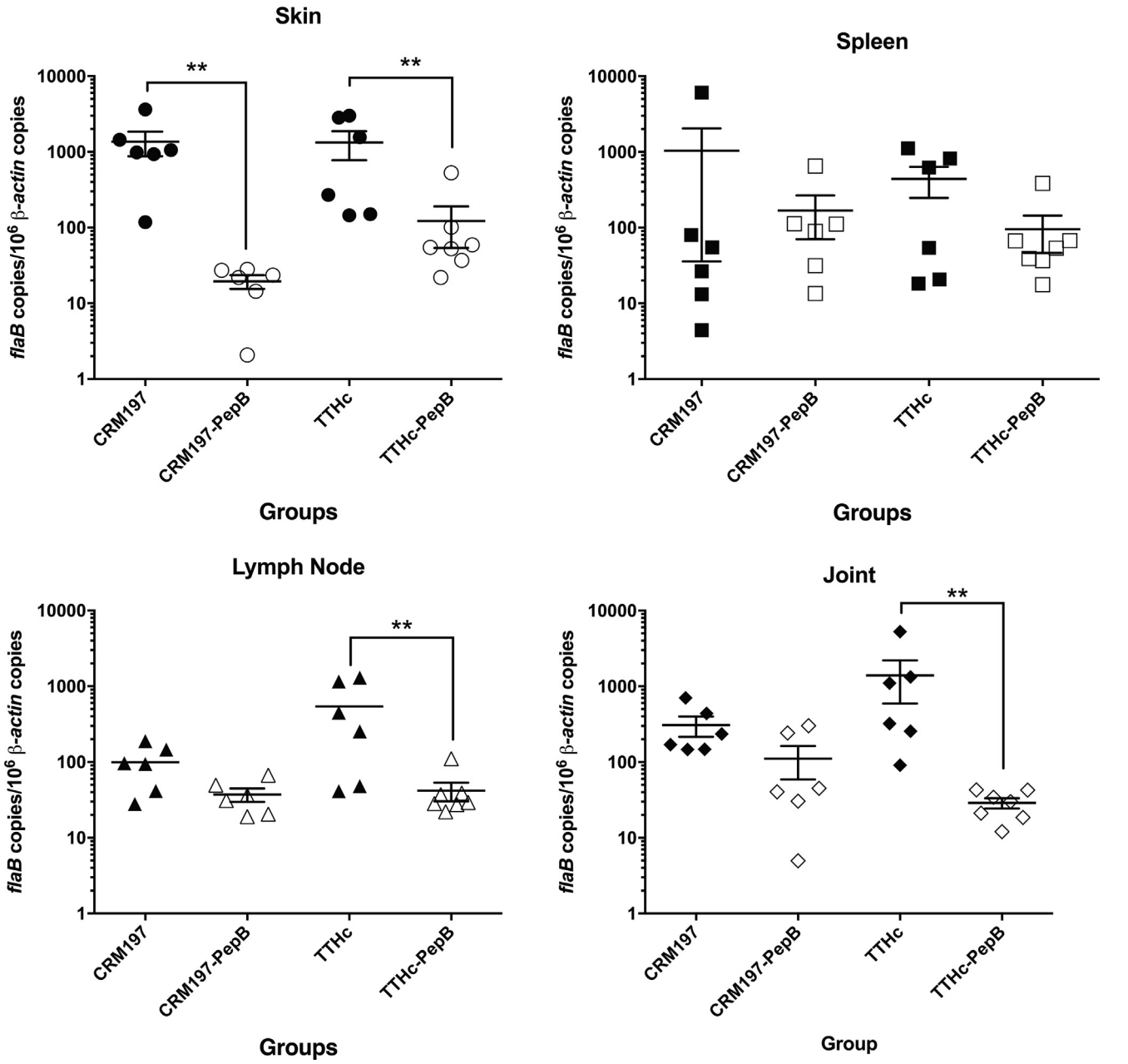

Fig. 3. TTHc:PepB conferred protection in immunized mice infected with *B. burgdorferi*. Bacterial burden was significantly reduced in mice immunized with TTHc:PepB in skin lymph nodes, and joints when compared with TTHc immunized animals. CRM197:PepB showed reduction in bacterial load, but it was only significantly reduced in skin when compared to CRM197. * Denotes statistical significances (* P value < 0.05; ** P value < 0.01) when compared with control groups.

Table 1
Recovery of *B. burgdorferi* from tissues sampled from immunized and control mice at 3 weeks post-needle infection.

Group/10^5 spirochetes/mouse	Skin	Lymph Node	Spleen	Bladder	Heart	Joint	Total
Control	4/4	4/4	4/4	4/4	4/4	4/4	4/4
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 (BR1)	6/6	6/6	6/6	6/6	6/6	6/6	36/36
TTHc:PepB (BR1)	2/7	2/7	2/7	2/7	2/7	2/7	12/42*
TTHc (BR2)	3/3	3/3	3/3	3/3	3/3	3/3	18/18
TTHc:PepB (BR2)	2/5	2/5	2/5	2/5	2/5	2/5	12/30*

No. of tissues positive/No. of tested tissues. BR1: Biological Replica 1; BR2: Biological replica 2.

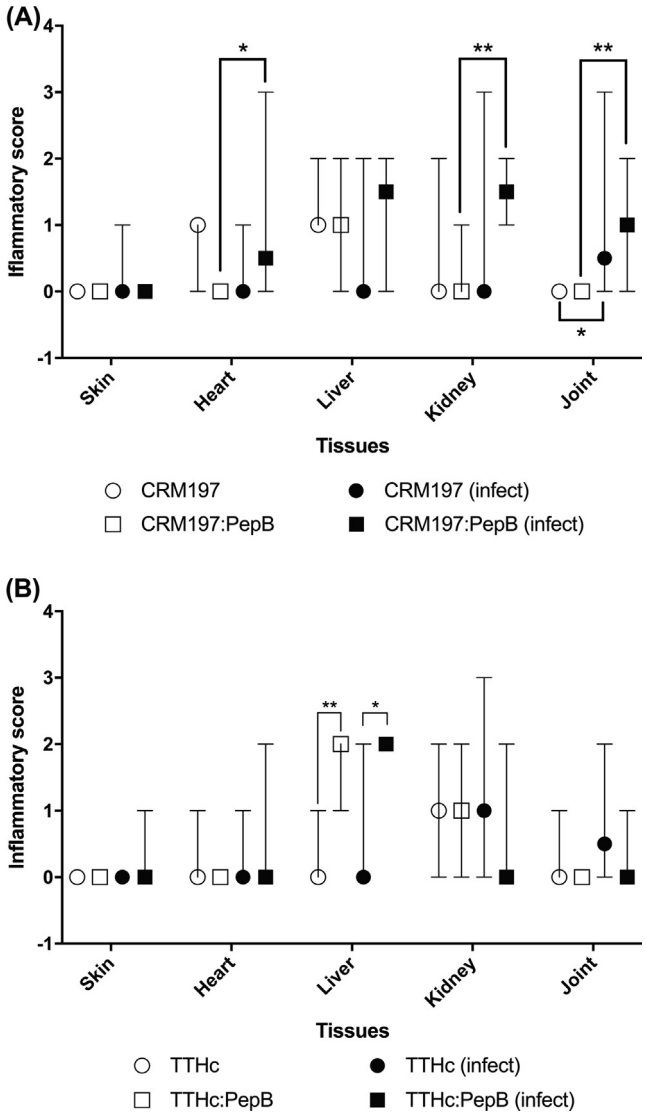

Fig. 4. Inflammatory scores in immunized animals at 8-weeks post-priming and 4-weeks post-infection using the subcutaneous route. (A) represents the inflammatory scores of tissues from animals immunized with CRM197 and CRM197:PepB. (B) represents the inflammatory scores of tissues from animals immunized with TTHc and TTHc:PepB * Denotes statistical significances (* *P* value < 0.05; ** *P* value < 0.01) when compared with control groups.

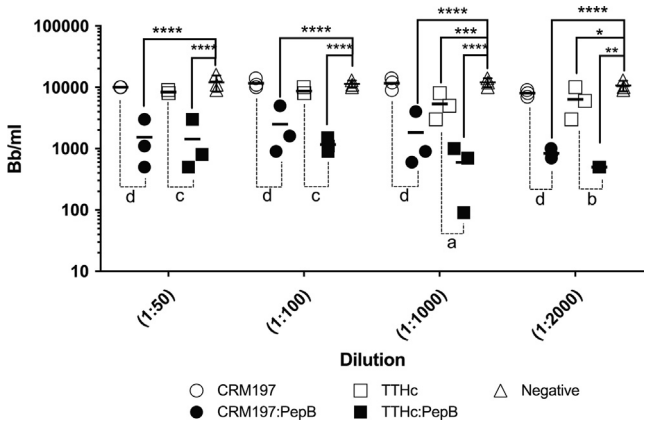

Fig. 5. Anti-PepB antibodies are Borrelicidal. *B. burgdorferi* cells (10^6/ml) were incubated with serial dilutions of sera from naïve and immunized animals in a 96-well plate for 1 h at 37 °C, followed by the addition of 1:10 dilution of commercially available rabbit complement. * Denotes statistical significance when compared with the negative (no serum) group (*P value < 0.05; ** P value < 0.01; *** P value < 0.001; **** 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).

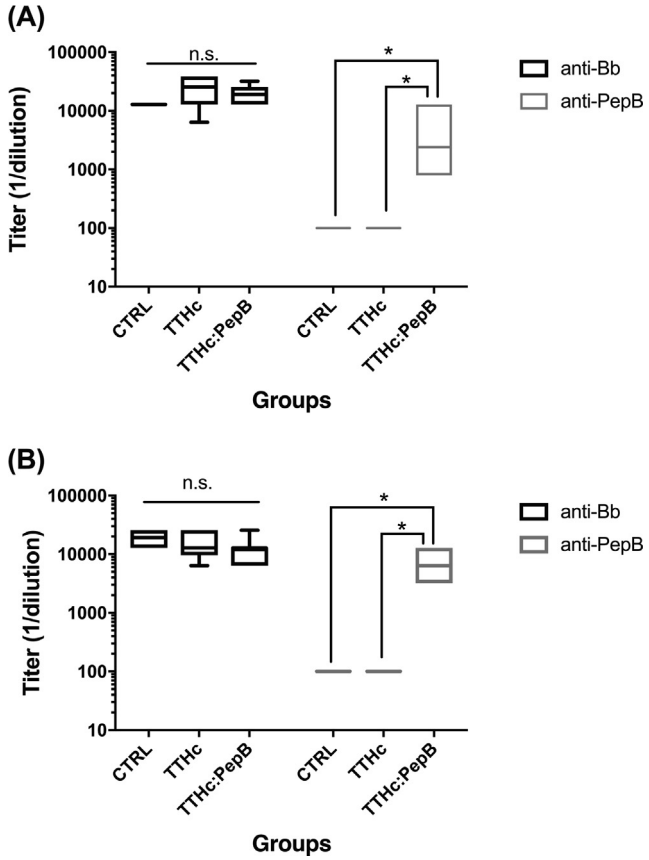

Fig. 6. Elevated IgG antibody titers were detected in immunized and control mice infected using the tick model. PepB (grey boxes) and *Borrelia burgdorferi* (black boxes) specific IgG were measured at 4 weeks post-tick challenge. Animals were infested with infected *Ixodes scapularis* ticks 8-weeks (A) or 12-weeks (B) post-priming. TTHc:PepB induced significant high anti-PepB IgG titers compared to the control groups. * Denotes statistical significances (* *P* value < 0.05) when compared with control groups; n.s.: denotes not significantly differences.

worth noticing that both after 8 and 12-weeks post-priming, there were animals that could clear more than 70% of the pathogen from tissues. No clear results were observed in lymph node tissues regardless on when animals were infested.

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 urinary bladders from 2 mice, and one spleen and tibiotarsal joint in the group received TTHc:PepB antigen (Supplemental Table S1). 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 (urinary bladder) out of 7 in TTHc:PepB group (Supplemental Table S2).

4. Discussion

The formulation of an effective vaccine candidate against LD in humans is of great importance since there is a lack of such vaccines in the market. However, extensive studies have mostly focused on evaluating the OspA and OspC as potential vaccine candidates for this disease [28,29]. In addition, few other novel antigens have

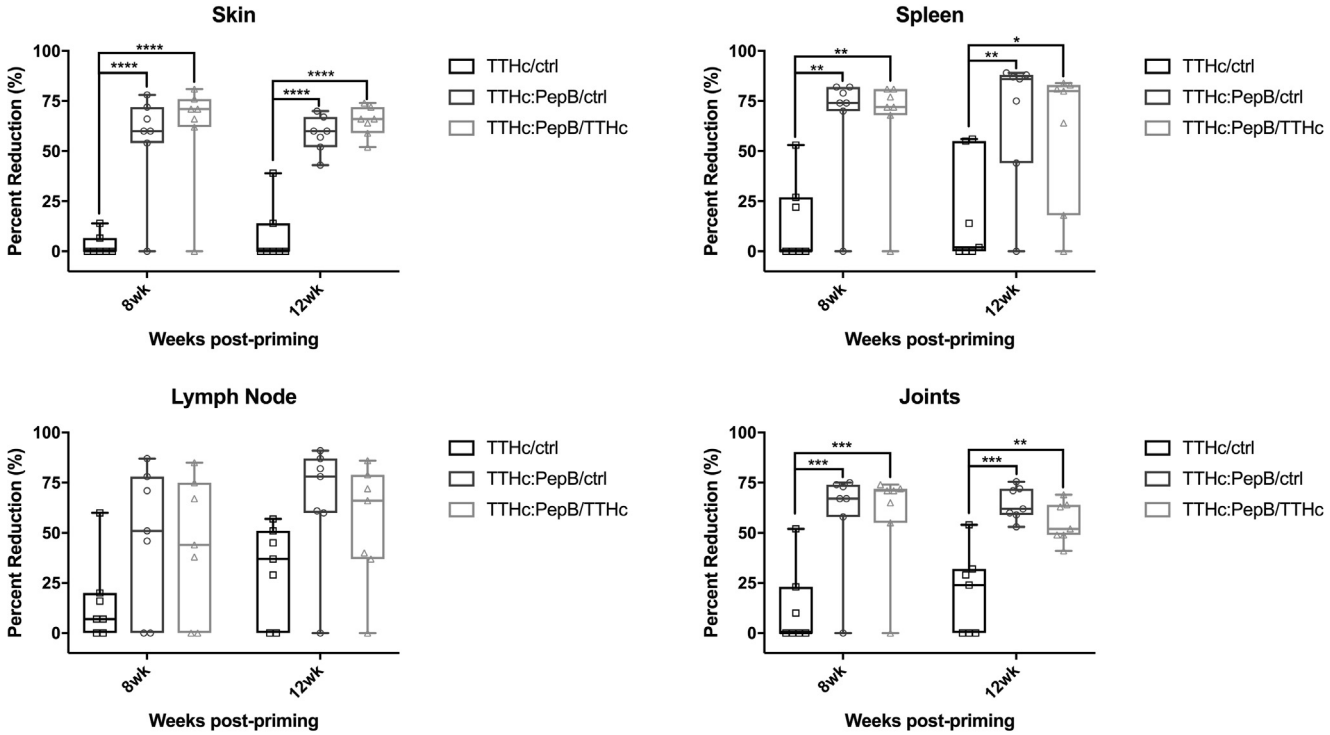

Fig. 7. TTHc:PepB immunized mice reduced up to 70% bacterial burden in tissues 4-weeks post-infestation with *Ixodes scapularis* ticks infected with *B. burgdorferi*. Animals were infested with *B. burgdorferi* infected *I. scapularis* at 8-weeks or 12-weeks post-priming. All tissues were dissected and bacterial burden was evaluated by quantitative real time PCR (qPCR) and normalized to *flaB* copies/10^6 β-acting copies. Numbers represented in this graph correspond to the percent bacterial burden reduction observed in vaccinated animals when compared with both control and TTHc groups. * Denotes statistical significances (* *P* value < 0.05; ** *P* value < 0.01; *** *P* value < 0.001; **** *P* value < 0.0001) when compared with control groups. No significant differences were observed in the percent reduction when comparing the same group at 8-weeks or 12 weeks post-priming.

been evaluated as vaccine candidates such as Decorin-binding protein A (DbpA) [30,31], and BBA52 [32].

Our team previously identified BB0172, as a potential vaccine candidate since it is exposed to the extracellular environment and binds to the human integrin $\alpha_3\beta_1$, via its von Willebrand factor A (vWFA) domain [19]. Furthermore, PepB, a BB0172 derived peptide, was able to induce protection in the murine model of LD when a dose $10 \times ID_{50}$ was administered to immunized animals with KLH-PepB antigens [18].

In the current study, we explored the protective efficacy of the PepB when conjugated with commercially available carrier molecules that have been approved for human use, as an effort to increase immunogenicity and protection induced by PepB. Based on a previous study [18], we sampled 3 animals from each group to evaluate antibody titers, and histopathological changes at 8-weeks post-priming. This time point was chosen since it correlated with the highest anti-PepB IgG titer reported in previous studies. It is known that humoral immune response is predominantly responsible for protection against infection with *B. burgdorferi* [33–35]. We previously performed passive transfer experiments to evaluate the involvement of the humoral immune response in protection against *B. burgdorferi* in animals immunized with PepB [18]. In the current manuscript we evaluated the serum bactericidal activity, which is mediated by antibody and complement reactions that lead to bacterial lysis. This is an effective indicator to evaluate functional activity of specific antibodies present in immunized animals [36]. In our study, we showed that conjugated PepB, regardless of the formulation used, was able to elicit production of borrelicidal antibodies. These findings are supported by other studies showing CRM197 and TTHc used in licensed conjugate meningococcal vaccines in Europe that induce robust bactericidal antibodies [36–38]. Such antibodies were able to confer protection against septicemia and meningitis caused by different strains of *Neisseria meningitis* [39]. However, in this study, the immune response was slightly skewed toward a Th1 response. In contrast, in our current study, the conjugated PepB formulations were able to elicit a mixed Th1/Th2 immune response, as determined by elevated IgG_1 and IgG_{2a} titers after needle infection in immunized mice. It is worth noting a slight predominance of Th1 immune response was observed, as indicated by the higher IgG_{2a} levels. This Th1 response has been reported as important to control LD spirochetes during infection in mice and humans [40–42]. These results contrast with our previous observations [18], where a Th2 response was induced when KLH was used as the carrier molecule. Under such condition, mice were only protected when infected using 10^3 Bb/mouse, while in our current study protection was observed at 10^5 Bb/mouse. Consequently, further studies will help elucidating the role that both IgG_1 and IgG_{2a} have in the control of *B. burgdorferi* infection in immunized animals using TTHc as promising carrier molecules.

Different approaches have been implemented to improve peptide efficacy such as use of scaffolding molecules, incorporation of additional immunostimulatory molecules, and the use of carrier proteins [43–45]. Of these strategies, the use of carrier molecules has been quite successful. In particular, the tetanus toxoid (TT) has been used extensively [46]. We included TTHc in our study since it is commercially available, and its precursor (TT) has already been licensed for human use [47]. The immunogenicity of TT is attributed to the H_C domain, which is the principle component of the TT molecule, as the toxin neutralizing epitopes are located within that domain [48,49]. Furthermore, it contains potent T-cell universal epitopes [50,51]. 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 follow-

ing challenge with tetanus toxin [52]. In addition, TTHc has been shown to activate specific $CD4^+$T cells including TH_1 cells [53,54].

In our study, the immune response showed a slight increase of IgG_{2a} titers compared to IgG_1 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, which showed 66% protection in immunized mice. Part of that mechanism might be attributed to the fact that in previous studies, murine IgG_{2a} isotype was found to be more effective in serum bactericidal assay than IgG_1 isotype, when they were tested against *Neisseria meningitidis*. Furthermore, in the same study, it was shown that IgG_{2a} was more efficient in opsonophagocytosis assays than IgG_1 [55].

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, compared to 9 in CRM197. This explanation could be supported by studies that illustrated that epitope density in a carrier protein is a key factor for the induction of a protective immune response [56–59]. In addition, a hapten density also has an effect on the selectivity and breadth of the induced antibody repertoire. High hapten density in a conjugate was able to produce broader spectrum antibodies than low hapten density construct [60]. Similar results were observed when comparing different meningococcal C conjugated vaccines [61,62].

After needle challenge with $1000 \times ID_{50}$, TTHc:PepB showed 66% protective efficacy in immunized mice, whereas CRM197:PepB was not protective. This protective efficacy was calculated by looking at bacterial clearance from tissues. Thus, the current results suggested that TTHc:PepB was the most efficacious vaccine candidate and we proceeded with that formulation to challenge immunized mice via the tick route of infection.

In the tick challenge, our results showed that, TTHc:PepB immunized animals were unable to clear the infection as previously observed after needle infection. Nevertheless, experiments demonstrate a significant reduction in the bacterial burden of infected animals immunized with TTHc:PepB, of up to 80% in some individuals. Therefore, the presented results are encouraging and deserve further investigation.

The laboratory adapted culture of *B. burgdorferi* used in this experiment showed all the essential plasmids, confirmed by PCR screening before using this strain in both the needle and tick challenge (Supplemental Fig. 2). In addition, there are several differences between cultured *B. burgdorferi* and spirochetes growing *in vivo* [63,64] that may affect results in the tick challenge. Based on that, it might 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. Another factor that could explain the results obtained in the tick model of infection that the inoculated dose of spirochetes through the tick bite is not controlled compared to the known dose administered via needle inoculation, and tick saliva proteins might have blocked the accessibility of specific antibodies to PepB antigen. Even though that is a plausible explanation, we were able to observe increased anti-PepB IgG levels in mice infected via the tick vector at both 8-weeks and 12-weeks post-priming, which suggests the accessibility of this antigen during early stages of infection.

It was previously shown that on week 12 post-priming, the anti-PepB antibody levels were reduced to basal levels [18]. 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 cells-dependent immune response, which may also account in the reduction of bacterial burden observed in the immunized animals. This is consistent with a previous study showing that $CD4^+$ T cells were required to control spirochetal load and amelioration of arthritis [65].

Immunized mice were challenged with infected ticks 8-weeks post-priming. It was showed that mostly all cultured tissues from the challenged mice were able to grow *B. burgdorferi*. However, anti-PepB antibodies reached their peak levels at 8-weeks post-priming, and they were ineffective to clear *B. burgdorferi* infection. Furthemore, similar results were observed following the tick challenge at 12-weeks post-priming.

During the tick feeding, the population of spirochetes are rapidly and highly expanded [66,67]. This contrast with a single injection of an infective dose used during needle challenge. It has been shown that administration of tick salivary gland extract is able to increase the dissemination and number of spirochetes in mice [68,69]. Therefore, the viable bacterial inoculum is highly increased at the tick bite site. Furthermore, spirochetes show higher virulence after they have been primed by a blood meal during tick feeding [70]. In addition, 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 [71]. Also, it facilitates survival of spirochetes within the infected host through binding to OspC lipoprotein [72].

In conclusion, TTHc:PepB formulation was able to induce protection in immunized mice compared to control group and those receiving CRM197:PepB. In addition, these antigen formulations did not induce major histopathological changes in immunized animals which makes them relatively safe for use in animal models. Moreover, when TTHc:PepB immunized animals were challenged using the tick model of transmission of *B. burgdorferi*, no pathogen clearance was observed; however, significant bacterial burden reduction in tissues such as skin, spleen and joint were reported. These observations suggest the potential use of PepB as a protective highly conserved antigen to control LD. In that regard, other approaches could be explored in order to increase protection and ensure clearance of pathogenic *B. burgdorferi* from vaccinated and infected animals.

Declaration of Competing Interest

The authors declare that they have no conflict of interest in the research presented in this study.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.07.092.

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