The Factor H-binding site of CspZ as a protective target against multi-strain, tick-transmitted Lyme disease

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ABSTRACT (144 words)

The spirochete *Borrelia burgdorferi* sensu lato is the causative agent of Lyme disease (LD). The spirochetes produce the CspZ protein that binds to a complement regulator, factor H (FH). Such binding downregulates activation of host complement to facilitate spirochete evasion of complement killing. However, vaccination with CspZ does not protect LD infection. In this study, we demonstrated that immunization with CspZ-YA, a CspZ mutant protein with no FH-binding activity, protected mice from infection by several spirochete genotypes introduced via tick feeding. We found that the sera from CspZ-YA-vaccinated mice more efficiently eliminated spirochetes and blocked CspZ FH-binding activity than sera from CspZ-immunized mice. We also found vaccination with CspZ, but not CspZ-YA, triggered the production of anti-FH antibodies, justifying CspZ-YA as a LD vaccine candidate. The mechanistic and efficacy information derived from this study provides insights into the development of a CspZ-based LD vaccine.

INTRODUCTION

Lyme disease (LD) is the most common vector-borne disease in both North America and Europe. In the United States, an estimated 300,000 individuals develop new cases each year, and in the past twenty years, the number of reported cases has drastically increased by over 200% (1). The Centers for Disease Control and Prevention (CDC) recently included LD in the top zoonotic disease of national concerns, with an annual economic burden over $700,000,000 in the United States (2). The only commercially-available human LD vaccine was withdrawn from the market nearly twenty years ago due to limited efficacy and perceived safety concerns, and there is still no human vaccine against the pathogenic spirochete that causes this disease, *Borrelia burgdorferi* sensu lato (3-5). Of all the Lyme borreliae, *B. burgdorferi* sensu stricto (hereafter *B. burgdorferi*), *B. garinii*, and *B. afzelii* are the three species that cause the majority of infections in North America, Europe, and Asia (1, 6). Lyme borreliae are transmitted to humans during the feeding of the hard tick vector *Ixodes*, after which the spirochetes colonize the feeding site of the skin and disseminate through the bloodstream to distal tissues and organs, causing symptoms including Erythema migrans, Acrodermatitis chronica atrophicans, arthritis, carditis, and neuroborreliosis (7, 8).

In order to traverse the bloodstream, Lyme borreliae must survive the vertebrate host innate immune defenses located in the blood, including complement (9-11)(for review, see (12-14)). This system is composed of three distinct pathways, the classical, the lectin, and the alternative pathway. The classical pathway is initiated by antigen-antibody interactions, the lectin pathway is activated after recognition of microbial carbohydrates, and the alternative pathway is triggered by the binding of complement protein C3b to the pathogen surface. All three pathways ultimately result in pathogen lysis or phagocytosis. To prevent self-damage of

human cells and tissues in the absence of microbial pathogens, complement is kept in check by a number of cell-associated and fluid phase complement regulatory proteins (CRP), including factor H (FH), which modulates the alternative pathway by inactivating C3b (12-14). Lyme borreliae encode several CRP-binding proteins (9-11), one of which is the outer surface FH-binding protein CspZ (15, 16). CspZ is not produced when spirochetes are in the tick vector, but rather is upregulated when spirochetes reside in mammalian hosts (17). When bound by CspZ, the FH regulatory activity is maintained, and complement activation is inhibited on the spirochete surface to promote infectivity (15, 18, 19).

While a minority of individual Lyme borreliae strains do not harbor *cspZ*, patients across North America and Europe with confirmed LD develop antibodies against CspZ, suggesting the strains that cause human infections produce this antigen (20-22). These results support previous findings that CspZ is present in multiple Lyme borreliae strains that cause human infection (20-24). Further, CspZ is highly conserved among different variants, even among those that do not bind human FH, emphasizing this protein as an attractive vaccine target (20, 21, 25). Surprisingly, vaccination with the wild type (WT) CspZ from *B. burgdorferi* strain B31 does not protect mice from spirochete colonization (22, 26, 27). We recently generated a CspZ mutant protein, CspZ-Y207A/Y211A (CspZ-YA, derived from that WT variant), and conjugated this protein to a virus-like particle (VLP). We found that this conjugated CspZ protein prevents infection in mice after needle inoculation of the *B. burgdorferi* B31-A3 (27). These findings elicit several intriguing questions: Does this regimen also protect from infections caused by tick-transmitted Lyme borreliae, and what is the protective mechanism that differentiates the efficacy between CspZ and CspZ-YA? In this study, we examined this CspZ-based vaccine in protecting

against multiple strains of tick-transmitted Lyme borreliae and elucidated the potential mechanisms that allow this vaccine to be efficacious against LD.

RESULTS

Vaccination with CspZ-YA but not CspZ prevented spirochete colonization and arthritis after tick transmission of *B. burgdorferi* strain B31-A3. We first examined the ability of the CspZ-YA vaccine to protect mice from tissue colonization of *B. burgdorferi* strain B31-A3 after tick feeding, the natural infection route of LD. Mice were inoculated and subsequently given two boosters of CspZ-YA or WT CspZ, or the same proteins conjugated to VLP (VLP-CspZ-YA or VLP-CspZ) (Supplementary Fig. 1). Mice inoculated with VLP or PBS were included as controls, as these offer no protection against *B. burgdorferi* infection (Supplementary Fig. 1). *I. scapularis* nymphal ticks carrying *B. burgdorferi* B31-A3 were allowed to feed on the immunized mice, and the levels of spirochete colonization were determined at 21 days post-feeding (dpf) (Supplementary Fig. 1).

As expected, PBS or VLP-inoculated mice fed on by nymphs carrying the *B. burgdorferi* strain B31-A3 had at least 30-fold higher spirochete burdens at the tick feeding site of the skin, bladder, knees, and ears than the negative control, PBS-inoculated mice fed on by naïve nymphs (Fig. 1). After being fed on by nymphs carrying the strain B31-A3, mice vaccinated with VLP-CspZ developed bacterial loads indistinguishable from those mice inoculated with CspZ in all tested tissues, and all tissues tested in both groups were at least 30-fold greater than the negative control (Fig. 1). These results suggest the CspZ conjugation to VLP is dispensable for preventing spirochete colonization caused by tickborne transmission, and also suggest the inability of CspZ as a vaccine to prevent spirochete colonization regardless of VLP conjugation. In contrast, after

nymphs carrying the strain B31-A3 fed on mice immunized with CspZ-YA or VLP-CspZ-YA, spirochete levels were indistinguishable from those in negative control mice for all tissues tested (Fig. 1). These results clearly indicate the efficacy of CspZ-YA as a vaccine to prevent spirochete colonization, as well as the unessential role of VLP conjugation of CspZ-YA to prevent spirochete colonization via tick transmission.

We also determined if vaccination with VLP-CspZ-YA or CspZ-YA prevents arthritis caused by tick-transmitted *B. burgdorferi* B31-A3. After immunizing and infecting mice (Supplementary Fig. 1), the tibiotarsus joints were histologically accessed for arthritic severity. The PBS- or VLP-inoculated mice reflected severe infiltration of inflammatory cells (indicated by arrows, Fig. 2a), whereas the negative control mice did not (Fig. 2a). Similarly, the joints from the mice immunized with VLP-CspZ or CspZ also showed substantial cellular infiltration both between and within the connective tissues and muscle (indicated by arrows, Fig. 2a). The arthritic severity of both mouse groups, as judged by the infiltration of inflammatory cells, was scored similarly (Fig. 2b). Conversely, the joints of the mice vaccinated with VLP-CspZ-YA or CspZ-YA displayed no detectable signs of inflammation, with the score indistinguishable from the negative control mice (Fig. 2b). Taken together, these results indicate that CspZ-YA vaccination prevents mice from LD-associated arthritis after tickborne transmission of spirochetes, and VLP conjugation of this antigen is irrelevant for this protection.

CspZ-YA vaccination protected mice from tissue colonization and arthritis caused by tickborne transmission of multiple Lyme borreliae strains. As VLP conjugation is dispensable for CspZ-YA in protecting against tickborne spirochete infection, we assessed whether unconjugated CspZ-YA protects against infection caused by additional Lyme borreliae

144 strains. Mice were immunized with CspZ-YA or PBS (control) in the same fashion as described,
145 followed by challenge with nymphal ticks carrying *B. burgdorferi* strain 297 or *B. afzelii* strain
146 VS461-JL (Supplementary Fig. 1). Both strains encode *cspZ* with 96 or 74% amino acid identity,
147 respectively, to the variant from the *B. burgdorferi* strain B31-A3 (Supplementary Fig. 2).
148 Whereas CspZ from the strain VS461-JL displayed human and mouse FH-binding activity
149 (Supplementary Fig. 3), CspZ from the strain 297 appears to not bind human or mouse FH (21,
150 22). Therefore, these strains were used as they represent pathogenic genotypes of Lyme borreliae
151 harboring CspZ variants with or without FH-binding activity.
152 At 21 days post initial immunization (dpii), the PBS-inoculated mice fed on by ticks
153 carrying *B. burgdorferi* strain 297 had at least 172-fold higher spirochete burdens than the PBS-
154 inoculated mice fed on by uninfected nymphs (negative control mice) at the tick feeding site of
155 the skin, bladder, knees, and ears (Fig. 3). These PBS-inoculated, strain 297-challenged mice
156 displayed apparent cellular infiltrations between the connective tissues and muscle of the
157 tibiotarsus joints, with arthritic scored as high as 2 (Fig. 4). In contrast, after being exposed to
158 ticks carrying the strain 297, the spirochete burdens of CspZ-YA-immunized mice were no
159 greater than the negative control mice for all tested tissues (Fig. 3). Moreover, no visual signs of
160 arthritis were identified in the CspZ-YA-immunized mice (Fig. 4). Similarly, the PBS-inoculated
161 mice fed on by nymphs carrying the strain VS461-JL had at least 261-fold greater bacterial loads
162 in all tested tissues than the negative control mice (Fig. 3). After exposure to nymphs harboring
163 the strain VS461-JL, the spirochete burdens in CspZ-YA-immunized mice were no different
164 from that in the negative control mice (Fig. 3). Surprisingly, the mice inoculated with PBS fed on
165 by ticks carrying the strain VS461-JL did not show any signs of arthritis, at levels similar to both
166 the negative control and CspZ-YA-immunized mice (Fig. 4). These results reflect the fact that

not every Lyme borreliae strain causes arthritis (28). Taken together, vaccination with CspZ-YA is capable of preventing tissue colonization by multiple spirochete strains, as well as alleviating arthritis caused by the strains that triggered such a manifestation.

Sera from CspZ-YA-immunized mice more efficiently eradicated multiple Lyme borreliae strains than sera from CspZ-vaccinated mice. To identify the protective mechanism of the CspZ-YA vaccine, we sought to determine the burdens of *B. burgdorferi* strains B31-A3 or 297, or *B. afzelii* strain VS461-JL in the fully engorged nymphs that fed on PBS- or CspZ-YA-inoculated mice. Compared to flat nymphs, spirochete burdens increased in ticks after feeding regardless of spirochete strain or mouse vaccination, as expected (Supplementary Fig. 4) (29). There was no difference in spirochete burdens in any replete nymphs regardless of vaccination or spirochete strain (Supplementary Fig. 4). In support of the fact that *cspZ* is not expressed when spirochetes reside in ticks (17), this result suggests that the CspZ-YA vaccination does not target spirochetes in nymphs as a protective mechanism.

We next compared the titers of anti-CspZ IgG and IgM (prior to tick feeding; Supplementary Fig. 1) in mice vaccinated with CspZ-YA or CspZ (which did not protect mice from infection). We found that the anti-CspZ IgM and IgG titers of were indistinguishable between these two groups, and these titers were 5- to 60-fold greater, respectively, than the negative control mice (Supplementary Fig. 5). These results are consistent with the previous observation that these proteins, when conjugated to VLP, induce similar levels of IgM and IgG against CspZ (27). We also determined the titers of different IgG subclasses in sera from CspZ- or CspZ-YA-immunized mice and detected 10- to 45-fold greater titers in compared to the negative control mouse sera (Supplementary Fig. 5). There was no difference between the levels

of IgG1, IgG2a, and IgG2b in the two vaccination groups (Supplementary Fig. 5). These results suggest that the quantity of antibodies against CspZ may not be the determining factor to differentiate the efficacy of CspZ and CspZ-YA immunization.

We further assessed the ability of sera from CspZ- or CspZ-YA-immunized mice to kill spirochetes *in vitro*. Both sera were capable of eradicating *B. burgdorferi* strain B31-A3; however, the CspZ-YA sera displayed 5.5-fold more efficient killing than the CspZ sera (Fig. 5a and D, and Supplementary Table 1). These results agree with our previous findings from mice immunized with these proteins conjugated to VLP (27). We further incubated those sera with *B. burgdorferi* strain 297 or *B. afzelii* strain VS461-JL. The sera from the CspZ-YA-vaccinated mice killed these strains 5- and 2.6-fold more efficiently than the sera from CspZ vaccinated mice, respectively (Fig. 5b, c, e, and f and Supplementary Table 1). These results indicate that the sera from CspZ-YA-vaccinated mice display superior spirochete killing compared to that from CspZ-immunized mice.

Sera from CspZ-YA but not CspZ-vaccinated mice blocked the CspZ FH-binding activity. We next examined whether there are differences in epitope recognition between the sera from mice immunized with CspZ-YA or CspZ. The fact that CspZ but not CspZ-YA binds to FH raises the possibility that the sera from mice immunized with the latter protein recognize epitopes within the CspZ FH-binding site and block the FH-binding activity. To test this possibility, we coated microtiter wells with CspZ and then incubated these wells with serially diluted sera from CspZ-YA-, CspZ-, or PBS-inoculated mice. CspZ-coated wells incubated with PBS were included as a control. Following the addition of human FH into the wells, the levels of FH immobilized by CspZ in each reaction were normalized to the control wells to determine the

213 percent FH binding (Fig. 6a). As expected, the levels of FH bound by CspZ were not impacted in
214 the presence of sera from the PBS-inoculated mouse serum (Fig. 6b). Whereas the addition of the
215 sera from CspZ-vaccinated mice did not reduce the levels of FH bound by CspZ, our results
216 clearly indicated that the sera from CspZ-YA-vaccinated mice decreased the levels of FH bound
217 to CspZ (50% inhibitory dilution rate: 1: 16,072 ± 1,595×; Fig. 6b). These results suggest that the
218 sera from CspZ-YA- but not CspZ-immunized mice contain antibodies that recognize the
219 epitopes within the CspZ FH-binding site.
220
221 **Immunization with CspZ and but not CspZ-YA generated antibodies against mammalian**
222 **FH.** A meningococcal vaccine based on a *Neisseria meningitis* FH-binding protein, fHBP,
223 triggers antibodies against FH from vaccinated individuals (30-32). To determine if a CspZ-
224 based vaccine also induces the production of anti-FH antibodies, we quantified the titers of IgG
225 that recognize human or mouse FH in the collected from the mice at 42dpii (prior to the
226 infection). As a negative control group, PBS-inoculated mice developed IgG against either FH
227 variant at titers close to the detection limits (limit of detection = 1 A.U.; Fig. 7). The sera from
228 CspZ-vaccinated mice had approximately 10-fold greater titers of anti-human or mouse FH IgG
229 than the negative control mice. However, the sera from CspZ-YA-immunized mice had titers
230 comparable to that from negative control mice (Fig. 7). These results indicate that immunization
231 with CspZ, but not CspZ-YA, triggers antibodies that recognize mammalian FH.
232
233 **DISCUSSION**
234 The route to introduce arthropod-borne pathogens into hosts often determines the infectivity
235 of these pathogens (33-35). Thus, an antigen that prevents infection via needle inoculation may

not necessarily protect against infections transmitted via vectors such as ticks (36, 37). In fact, we previously reported that VLP conjugation enhances the efficacy of CspZ-based antigens in protecting mice after needle inoculation of *B. burgdorferi* (27). In contrast, we showed in the current study that such a regimen is dispensable for those CspZ proteins in preventing LD-associated spirochete colonization through tick transmission. Such disagreement of vaccine efficacy could be due to the fundamental differences between infection by different routes (e.g., the dosage of spirochetes, the presence of tick-specific proteins, and differential surface protein expression spirochetes cultivated *in vitro* and derived from ticks).

In this report, we observed that CspZ-YA vaccination of mice prevents infection caused by ticks carrying different Lyme borreliae strains. This cross-protection via such a physiologically relevant infection route highlights the feasibility of using CspZ-YA as a LD vaccine. It should be noted that we used *I. scapularis* as a model to examine the efficacy of the CspZ-YA vaccine, allowing us to attribute any differences of efficacy to particular spirochete strains or species. However, the caveat is that some Lyme borreliae (e.g., *B. afzelii*) are often isolated from *I. ricinus* but not *I. scapularis* ticks (1), and vector adaption appears to contribute to the transmission efficiency of spirochetes (38-40). Further investigation in the impact of such a difference to the efficacy of CspZ-YA vaccine would be warranted.

Our previous findings that the passive immunization with sera from CspZ-YA- but not CspZ-vaccinated mice prevents *B. burgdorferi* colonization indicate an antibody-dependent protective mechanism for this antigen (27). Consistent with our previous finding using needle infection of Lyme borreliae, CspZ-YA but not CspZ vaccination protects mice from colonization and LD-associated arthritis via tick feeding. These results suggest the efficacy of a CspZ-based vaccine is dependent on mutating the FH-binding site. FH is present in human blood at

concentrations as high as 600 μg ml^{-1} (41). A physiological range of CspZ's affinity in binding to mammalian FH (K_D ~10^{-7} M) suggests a tight association of both proteins upon vaccination. Such interactions would then prevent the exposure of epitopes within the CspZ FH-binding site. Thus, one model to address the finding of CspZ-YA as a more efficacious vaccine than CspZ is that the antibodies induced by former but not latter antigen compete with FH to bind to such epitopes, resulting in the blocking of the spirochetes' FH-binding-mediated complement evasion. In fact, our finding that the sera from CspZ-YA-immunized mice block the binding of FH to CspZ in a dose-dependent manner clearly supports this possibility. It is noteworthy that polyclonal sera from CspZ-YA-immunized mice are used in this study, and 1: 100× dilution of those sera nearly completely inhibited the binding of FH to CspZ. This result suggests that the undiluted sera with physiological relevant amount/concentration of anti-CspZ antibodies would also be capable of inhibiting FH binding to CspZ. Additionally, CspZ-mediated FH-binding activity is allelically variable, despite 98% identity among variants (22). One variant used in this study (CspZ from *B. burgdorferi* strain 297) appears to not bind to mammalian FH (21, 22), but CspZ-YA-immunized mice were protected from the infection by this spirochete strain. Sera from CspZ-YA-vaccinated mice eliminated *B. burgdorferi* strain 297 more efficiently than sera from CspZ-immunized mice. Taken together, these results thus suggest an additional protective mechanism of CspZ-YA: The resulting antibodies that recognize the epitopes within FH-binding site trigger bacterial killing through the classical pathway.

We found that the sera from CspZ- but not CspZ-YA-vaccinated mice recognize human and mouse FH, suggesting that the CspZ but not CspZ-YA triggers the production of anti-FH antibodies. A previous study indicates that FH's conformation alters upon its binding by a *S. pneumoniae* antigen, PspC (42). These observations lead to a possibility that the formation of a

FH neoepitope in the binding interface with CspZ contributes to the induction of anti-FH antibodies. Such antibodies in CspZ-vaccinated mice may prevent FH from downregulating complement, which may exhaust C3 and reduce complement-mediated bactericidal activity, resulting in inefficacious protection of the CspZ vaccination (43, 44{Jokiranta, 1999 #3871, 45). Thus, the lack of anti-FH antibodies in CspZ-YA vaccinated mice may prevent the exhaustion of complement components, allowing this vaccine to be efficacious. This notion warrants future studies. Further, our finding of anti-FH antibodies induced after CspZ vaccination also leads to another intriguing but unanswered question: Do those antibodies promote manifestations mediated by autoimmune responses? In fact, a *Neisseria meningitidis* FH-binding protein, Fhbp, has been used clinically as a human meningococcal vaccine, and humans develop anti-FH antibodies after vaccination. To date, no autoimmune manifestations have been attributed to such antibodies, possibly due to transient and low levels of these antibodies (30). In spite of that, the possibility of the autoimmunity triggered by anti-FH antibodies from CspZ vaccination still cannot be excluded, emphasizing the benefit in opting for CspZ-YA as human vaccines from a safety perspective.

In summary, we used a tick infection model to demonstrate the efficacy of vaccination with CspZ-YA, an antigen previously shown to prevent *B. burgdorferi* colonization and arthritis after needle inoculation. This antigen-mediated prevention can also protect against infections caused by tickborne transmission of other spirochete strains or species that can cause human LD. In addition, we elucidated the potential protective mechanisms of this antigen as well as tested its safety by detecting the levels of anti-FH antibodies. The mechanistic and efficacy information derived from this study will provide insights into antigen engineering to develop a vaccine for LD and can be extended to other pathogens.

METHODS

Ethics Statement. All mouse experiments were performed in strict accordance with all provisions of the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the PHS Policy on Humane Care and Use of Laboratory Animals. The protocol (Docket Number 16-451 and 19-451) was approved by the Institutional Animal Care and Use Agency of Wadsworth Center, New York State Department of Health. All efforts were made to minimize animal suffering.

Mouse, ticks, and bacterial strains. Three-week-old, female C3H/HeN mice were purchased from Charles River (Wilmington, MA, USA). This mouse strain was utilized because it develops LD manifestations (e.g., arthritis) during *B. burgdorferi* infection and thus is commonly used to test the efficacy of LD vaccines (46, 47). BALB/c C3-deficient mice from in-house breeding colonies were used for generating infected ticks (19, 29). *Ixodes scapularis* tick larvae were obtained from BEI Resources (Manassas, VA).

Escherichia coli strain BL21(DE3) and derivatives were grown at 37°C in Luria-Bertani (BD Bioscience, Franklin Lakes, NJ) broth or agar, supplemented with kanamycin (25µg/mL), ampicillin (100µg/mL), or no antibiotics when appropriate. *Borrelia* strains were grown at 33°C in BSK II complete medium (48) (Supplementary Table 2). Cultures of *B. burgdorferi* strain B31-A3 was tested with PCR to ensure a full plasmid profile prior to use (49, 50) whereas *B. burgdorferi* strain 297 and *B. afzelii* strain VS461-JL were maintained as fewer than 10 passages.

Generation of recombinant proteins and vaccines. The genomic DNA of *B. afzelii* strain VS461 provided by Dr. John Leong (denoted "VS461-JL") (Supplementary Fig. 2) and Dr. Peter Kraiczy (denoted "VS461-PK") (Supplementary Fig. 2) was used to amplify *cspZ*. The DNA sequences encoding *cspZ* were amplified and sequenced by PCR using primers "BspA14s-CspZ-Fwd" and "BspA14s-CspZ-Rev" (Supplementary Table 3; NCBI accession MN809989 and MN809990). The sequences of both alleles were identical (Supplementary Fig. 2). The open reading frames of *cspZ* from the strain VS461-JL lacking the putative signal sequences were amplified (residues 24 to 280, Supplementary Table 3) and cloned into pGEX-4T-2 at the *BamH*I and *Sal*I sites (GE Healthcare, Piscataway, NJ) (19, 27). This plasmid was transformed into *E. coli* strain BL21(DE3) and the plasmid insert was sequenced (Wadsworth ATGC Core Facility, NYS Department of Health, Albany, NY, USA). The glutathione-S-transferase GST-tagged CspZ (GST-CspZ) variants from the strains VS461-JL or B31-A3 were produced and purified by glutathione chromatography according to the manufacturer's instructions (BD Bioscience, Franklin Lakes, NJ) (27).

Recombinant CspZ and CspZ-YA from the strain B31-A3 were purified for mouse vaccination with HisTrap FF column (GE Healthcare, Chicago, IL, USA) from the *E. coli* strain BL21(DE3) containing pETm_11 encoding CspZ from the strain B31-A3 (residues 21–236), or an altered open reading frame encoding CspZ-Y207A/Y211A (residues 21–236 with tyrosine-207 and -211 replaced by alanine), followed by a TEV protease cleavage site (Supplementary Table 3). These proteins were conjugated to purified VLP Qβ with SMPH (succinimidyl-6-[(β-maleimidopropionamido) hexanoate]) at N-terminus of the protein as described (27).

Generation of infected ticks. Generating infected *I. scapularis* ticks has been described previously (46). Basically, BALB/c C3-deficient mice were infected subcutaneously with 10^5 of the strains B31-A3, 297, or VS461-JL (19, 29). Ear tissues were collected via ear punch, and bacterial gDNA was purified for detection with qPCR to confirm infection (see section "Quantification of spirochete burden"). Approximately 100 to 200 uninfected larvae were then allowed to feed to repletion on the infected mice as described previously (46). The engorged larvae were collected and allowed to molt into nymphs in a desiccator at room temperature with 95% relative humidity and light dark control (light to dark, 16:8 hours).

Mouse immunization and infection. Mice were vaccinated as described, with slight modifications (27). Fifty µl of PBS (control) or 25µg of VLP, CspZ, VLP-CspZ, CspZ-YA, VLP-CspZ-YA in 50µl of PBS was thoroughly mixed with 50µl TiterMax Gold adjuvant (Norcross, GA, USA). C3H/HeN mice were immunized subcutaneously with 100µl of the vaccine. Mice received boosters of the same composition at 14 and 28 dpii for a total of three immunizations over six weeks. Forty-two dpii, blood was collected via submandibular bleeding to isolate serum (Supplementary Fig. 1). At 49 dpii, uninfected (control) or infected flat nymphs were placed in a chamber on the immunized or PBS-inoculated C3H/HeN mice as described (Supplementary Fig. 1)(26). Five nymphs were allowed to feed to repletion on each mouse, and a subset of nymphs was collected pre- and post-feeding. DNA was purified from these nymphs, and spirochetes were quantified following parameters in the section "Quantification of spirochete burdens".

Quantification of spirochete burdens. To quantify spirochete burdens, mice were sacrificed at 21 dpf and the tick feeding site of the skin, knees, bladder, and ears were collected (Supplementary Fig. 1). DNA was purified using EZ-10 Spin Column Animal Genomic DNA Mini-Prep Kit (Bio Basic, Inc., Markham, Ontario, CA). Spirochete burdens were quantified based on the amplification of *16srRNA* (Supplementary Table 4) with qPCR using an Applied Biosystems 7500 Real-Time PCR system (ThermoFisher) in conjunction with PowerUp™ SYBR® Green Master Mix (ThermoFisher) as described (27, 51). The number of *16srRNA* copies was calculated by establishing a threshold cycle (Cq) standard curve of a known number of *16srRNA* gene extracted from strain B31-A3, and burdens were normalized to 100ng of total DNA.

Histological analysis of LD-associated arthritis. Mice were sacrificed at 21 dpf, and tibiotarsus joints were collected to assess arthritis via tissue histopathology. Tissues were fixed, decalcified, and prepared as slides stained with hematoxylin and eosin as described previously (Wadsworth Histopathology Core Facility, NYS Department of Health, Albany, NY, USA) (27). At least ten sections per mouse were blindly evaluated for signs of arthritis using histological parameters for *Borrelia*-induced inflammation and scored as described (51). The image was scored based on the severity of the inflammation as 0 (no inflammation), 1 (mild inflammation with less than two small foci of infiltration), 2 (moderate inflammation with two or more foci of infiltration), or 3 (severe inflammation with focal and diffuse infiltration covering a large area).

ELISAs. An ELISA to determine the human or mouse FH-binding ability of CspZ was performed as described (19, 52). In brief, human (ComTech, Tyler, TX) or mouse

(MyBiosource, San Diego, CA) FH was coated onto microtiter plate wells. Serially diluted GST (negative control) or GST-tagged CspZ from the strains B31-A3 or VS461-JL was added to the wells. The binding of GST-tagged proteins was detected using mouse anti-GST tag (ThermoFisher, Waltham, MA; 1:200×) and horseradish peroxidase-conjugated goat anti-mouse IgG (ThermoFisher; 1:1,000×). Tetramethylbenzidine solution (ThermoFisher) was added to each well and incubated for five minutes, then the reaction was stopped with hydrosulfuric acid. Plates were read at 405nm using a Tecan Sunrise Microplate reader (Tecan, Morrisville, NC). To determine the dissociation constant (K_D), the data were fitted with Equation 1 using GraphPad Prism software (GraphPad, La Jolla, CA).

$$OD405 = \frac{OD405max\ [CspZ\ proteins]}{KD + [CspZ\ proteins]} \quad \text{(Equation 1)}$$

To determine the titers of anti-CspZ antibodies, 50µl of serially diluted mouse serum (1:100×, 1:300×, 1:900×) from 42 dpii was added to microtiter wells coated with recombinant CspZ. Total IgG and IgM were detected using HRP-conjugated goat anti-mouse IgM and IgG, respectively (1:20,000×; Bethyl, Montgomery, TX, USA). The IgG subclasses were detected using HRP conjugated goat anti-mouse IgG1, IgG2a, IgG2b (1:8,000×, SouthernBiotech, Birmingham, AL). To measure the levels of anti-FH antibodies in the same vaccinated mouse sera, one µg of human or mouse FH was coated on microtiter wells and then incubated with sera, followed by the addition of conjugated goat anti-mouse IgG (1:20,000×; Bethyl). After the addition of antibodies, tetramethyl benzidine solution (ThermoFisher) was added, and the absorbance detected at 620nm for 10 cycles of 60 second kinetic intervals with 10 seconds shaking duration in a Sunrise absorbance ELISA plate reader (Tecan, Männedorf, Switzerland). For each serum sample, the maximum slope of optical density/minute of all the dilutions was

multiplied by the respective dilution factor, and the greatest value used as representative of antibody titers (arbitrary unit (A.U.)).

To examine the ability of antibodies to block the FH-binding activity of CspZ, one μg of mouse anti-GST IgG (ThermoFisher) was coated on ELISA plate wells, followed by incubation with one μM of recombinant GST-CspZ. After blocking with 5% BSA in PBS buffer, the wells were incubated with PBS (control) or serially-diluted mouse sera collected at 42 dpii (1:200×, 1:600×, 1:1800×, 1:5400×, 1:16200×, 1:48600×, 1:145800×, and 1:437400×) followed by being mixed with one μM of human FH. Sheep anti-human FH (1:200×, ThermoFisher) and then goat anti-sheep HRP (1:2000×, ThermoFisher) were added, and the levels of FH binding were detected as mentioned above. Data were expressed as the proportion of FH binding from serum-treated to PBS-treated wells. The 50% inhibitory dilution, representing the serum dilution rate that blocks 50% of FH binding, was calculated using dose-response stimulation fitting in GraphPad Prism 5.04.

Borreliacidal assays. Mouse sera collected at 42 dpii were used to determine the bactericidal activity against *B. burgdorferi* as described (27). Briefly, these mouse sera were heat-treated to inactivate complement, serially diluted, and mixed with complement-preserved guinea pig serum (Sigma-Aldrich, St. Louis, MO) or heat-inactivated guinea pig serum (negative control). After adding the strains B31-A3, 297, or VS461-JL, the mixture was incubated at 33°C for 24 hours. Surviving spirochetes were quantified by directly counting the motile spirochetes using dark-field microscopy and expressed as the proportion of serum-treated to untreated Lyme borreliae. The 50% borreliacidal titer, representing the serum dilution rate that kills 50% of spirochetes, was calculated using dose-response stimulation fitting in GraphPad Prism 5.04.

Statistical analyses. Significant differences were determined with a Mann-Whitney test (between two groups) or Kruskal-Wallis test with Dunn's multiple comparisons (more than two groups) using GraphPad Prism 5.04. A p-value < 0.05 was used to determine significance.

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FIGURE LEGENDS

Figure 1. Vaccination of VLP-CspZ-YA or CspZ-YA protected mice from borrelial tissue colonization caused by tick feeding. Five PBS-, six VLP-CspZ-, VLP-CspZ-YA-, or CspZ-YA- or seven VLP- or CspZ-inoculated C3H/HeN mice were fed on by nymphs carrying *B. burgdorferi* strain B31-A3. Uninfected nymphs were placed on an additional five mice inoculated with PBS as negative control ("N.A."). Spirochete burdens at **(a**) the tick feeding site ("Inoc. Site"), **(b)** knees, **(c)** bladder, and **(d)** ears were quantitatively measured at 21 dpf, shown as the number of spirochetes per 100ng total DNA. Data shown are the geometric mean ± geometric standard deviation of the spirochete burdens from each group of mice. Asterisks indicate the statistical significance ($p < 0.05$, Kruskal Wallis test with Dunn's multiple comparisons) of differences in bacterial burdens relative to uninfected mice.

Figure 2. Vaccination of VLP-CspZ-YA or CspZ-YA protected mice from LD-associated arthritis caused by tick feeding. Five PBS-, six VLP-CspZ-, VLP-CspZ-YA-, or CspZ-YA- or seven VLP- or CspZ-inoculated C3H/HeN mice were fed on by nymphs carrying *B. burgdorferi* strain B31-A3. Uninfected nymphs were placed on an additional five mice inoculated with PBS as negative control ("N.A."). Tibiotarsus joints were collected from these mice at 21 dpf. To assess inflammation, tissues were fixed and stained with hematoxylin and eosin. **(a)** Representative images from one mouse per group are shown. Top panels are lower-resolution images (joint, ×10 [bar, 160 μm]); bottom panels are higher-resolution images (joint, 2×20 [bar, 80 μm]) of selected areas (highlighted in top panels). Arrows indicate infiltration of immune cells. **(b)** To quantitate inflammation of joint tissues, at least ten random sections of tibiotarsus joints from each mouse were scored on a scale of 0-3 for the severity of arthritis. Data shown are

660 the mean inflammation score ± standard deviation of the arthritis scores from each group of
661 mice. Asterisks indicate the statistical significance ($p < 0.05$, Kruskal Wallis test with Dunn's
662 multiple comparisons) of differences in inflammation relative to uninfected mice.
663
664 **Figure 3. Immunization with CspZ-YA prevented tissue colonization after exposure of tick**
665 **carrying multiple Lyme borreliae strains.** Five C3H/HeN mice were vaccinated with CspZ-
666 YA or inoculated with PBS and subsequently infected by nymphal ticks carrying *B. burgdorferi*
667 strain 297 or *B. afzelii* strain VS461-JL. Uninfected nymphs were placed on an additional five
668 mice inoculated with PBS as negative control ("N.A."). Spirochete burdens at **(a)** the tick
669 feeding site ("Inoc. Site"), **(b)** knees, **(c)** bladder, and **(d)** ears were quantitatively measured at 21
670 dpf, indicated as the number of spirochetes per 100ng total DNA. Data shown are the geometric
671 mean ± geometric standard deviation of the spirochete burdens from each group of the mice.
672 Asterisks indicate the statistical significance ($p < 0.05$, Kruskal Wallis test with Dunn's multiple
673 comparisons) of differences in bacterial burden relative to uninfected mice.
674
675 **Figure 4. Immunization with CspZ-YA prevented LD-associated arthritis after exposure of**
676 **tick carrying multiple Lyme borreliae strains.** Five C3H/HeN mice were vaccinated with
677 CspZ-YA or inoculated with PBS and subsequently infected by nymphal ticks carrying *B.*
678 *burgdorferi* strain 297 or *B. afzelii* strain VS461-JL. Uninfected nymphs were placed on an
679 additional five mice inoculated with PBS as negative control ("N.A."). Tibiotarsus joints were
680 collected from these mice at 21 dpf. To assess inflammation, tissues were fixed and stained with
681 hematoxylin and eosin. **(a)** Representative images from one mouse per group are shown. Top
682 panels are lower-resolution images (joint, ×10 [bar, 160 μm]); bottom panels are higher-

683 resolution images (joint, 2×20 [bar, 80 μm]) of selected areas (highlighted in top panels). Arrows
684 indicate infiltration of immune cells. **(b)** To quantitate inflammation of joint tissues, at least ten
685 random sections of tibiotarsus joints from each mouse were scored on a scale of 0-3 for the
686 severity of arthritis. Data shown are the mean inflammation score ± standard deviation of the
687 arthritis scores from each group of mice. Asterisks indicate the statistical significance ($p < 0.05$,
688 Kruskal Wallis test with Dunn's multiple comparisons) of differences in inflammation relative to
689 uninfected mice.
690
691 **Figure 5. Sera from mice immunized with CspZ-YA had more robust levels of borreliacidal**
692 **activity than that from CspZ-vaccinated mice.** Sera from five C3H/HeN mice inoculated with
693 CspZ, CspZ-YA, or PBS (negative control) were obtained at 42 dpii. Sera were serially diluted
694 as indicated, and mixed with guinea pig complement and **(a and d)** *B. burgdorferi* strains B31-
695 A3 or **(b and e)** 297, or **(c and f)** *B. afzelii* strain VS461-JL (5×10^5 cells ml^{-1}). After being
696 incubated for 24 hours, surviving spirochetes were quantified from three fields of view for each
697 sample using dark-field microscopy. The work was performed on three independent experiments.
698 **(a to c)** The survival percentage was derived from the proportion of serum-treated to untreated
699 spirochetes. Data shown are the mean ± SEM of the survival percentage from three replicates in
700 one representative experiment. **(d to f)** The 50% borreliacidal dilution of each serum sample,
701 representing the dilution rate that effectively killed 50% of spirochetes, was obtained from curve-
702 fitting and extrapolation of Panel A to C. Data shown are the mean ± SEM of the borreliacidal
703 titers from three experiments. The exact values are shown in Supplementary Table 1. PBS-
704 vaccinated mouse sera displayed no bactericidal activity ("NK", no killing). Statistical

significance ($p < 0.05$, Mann-Whitney test) of differences in borreliacidal titers between groups are indicated ("#").

Figure 6. Sera from CspZ-YA but not CspZ-vaccinated mice blocked FH binding to CspZ. Sera from five C3H/HeN mice inoculated with CspZ, CspZ-YA, or PBS (negative control) were obtained at 42 dpii. **(a)** A schematic diagram shows the experimental setup. GST-CspZ was added to a microtiter plate coated with anti-GST, in order to orient CspZ with the FH-binding site exposed. Wells were treated with PBS or indicated dilution rates of mouse sera and then incubated with human FH. The levels of bound FH were quantified using sheep anti-human FH and goat anti-sheep HRP IgG as primary and secondary antibodies, respectively. The work was performed on three independent experiments; within each experiment, samples were run in triplicate. **(b)** Data are expressed as the percent FH binding, derived by normalizing the levels of bound FH from mouse sera treated wells to that in PBS-treated wells. Data shown are the mean ± SEM of the percent FH binding from three replicates in one representative experiment. The dilution rate of the sera to inhibit 50% of FH bound by CspZ was obtained from curve-fitting and extrapolation of Panel A (50% inhibitory dilution rate: 1: 16,072 ± 1,595 ×).

Figure 7. Vaccination with CspZ triggered antibodies against FH. Sera from five C3H/HeN mice inoculated with CspZ, CspZ-YA, or PBS (negative control) at 42 dpii were incubated with microtiter plate wells coated with human or mouse FH. Mouse IgG bound to FH were detected using goat anti-mouse IgG. Data shown are the geometric mean ± geometric standard deviation of antibody titers from each group of the mice. Significant differences ($p < 0.05$, Mann-Whitney

test) in the levels of antibody titers relative to sera from PBS-inoculated mice ("*") or between two groups relative to each other ("#") are indicated.