Non-anticoagulant heparin as a pre-exposure prophylaxis prevents Lyme disease infection.

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ABSTRACT: Lyme disease (LD) is caused by the spirochete *Borrelia burgdorferi* sensu lato (*Bbsl*). After transmission to humans by ticks, *Bbsl* spreads to multiple organs, leading to arthritis, carditis, and neuroborreliosis. No effective prophylaxis against human LD prior to tick exposure is currently available. Thus, a pre-exposure prophylaxis (PrEP) against LD is needed. The establishment of LD bacteria at diverse sites is dictated partly by the binding of *Bbsl* to proteoglycans (PGs) and glycosaminoglycans (GAGs) in tissues. The drug heparin is structurally similar to these GAGs and inhibits *Bbsl* attachment to PGs, GAGs, cells, and tissues, suggesting its potential to prevent LD. However, the anticoagulant activity of heparin often results in hemorrhage, hampering the development of this compound as LD PrEP. We have previously synthesized a non-anticoagulant version of heparins (NACHs), which was verified for safety in mice and humans. Here, we showed that NACH blocks *Bbsl* attachment to PGs, GAGs, and mammalian cells. We also found that treating mice with NACH prior to the exposure of ticks carrying *Bbsl* followed by continuous administration of this compound prevents tissue colonization by *Bbsl*. Furthermore, NACH-treated mice develop greater levels of IgG and IgM against *Bbsl* at early stages of infection, suggesting that the upregulation of antibody immune responses may be one of the mechanisms for NACH-mediated LD prevention. This is one of the first studies examining the ability of a heparin-based compound to prevent LD prior to tick exposure. The information presented might also be extended to prevent other infectious diseases agents.

KEYWORDS: Lyme disease, Borrelia, heparin, NACH, Pre-exposure prophylaxis

INTRODUCTION

Transmitted by *Ixodes* ticks, Lyme disease is the most common vector-borne disease in the northern hemisphere[1-2]. This disease is caused by multiple species of the spirochete *Borrelia burgdorferi* sensu lato. In North America, the majority of human Lyme disease cases is caused by one species of these spirochetes, *B. burgdorferi* sensu stricto (*B. burgdorferi*) [3] whereas another species, B. mayonii, was recently identified to also cause human Lyme disease in US [4]. A 2018 CDC study found that U.S. tickborne disease cases doubled from 2004-2016, with Lyme disease accounting for 82% of all reports[5]. Acute illness is commonly treated with antibiotics, but prolonged disease manifestations cost the U.S. health care system close to $1.3 billion a year (approximately $3,000 per patient on average)[6]. However, no effective Lyme disease prevention used prior to exposure to ticks is currently available[7]. Upon the tick bite, Lyme borreliae establish infection at bite site in skin and disseminate through the bloodstream to distal tissues and organs, leading to manifestations, including arthritis, carditis, and neuroborreliosis [3, 8]. Such clinical observations support that spirochete attachment to host tissues is a requirement for the development of these manifestations [9-10].

Vertebrate hosts generate multiple receptors on cell surface that are exploited by microbes including Lyme borreliae to facilitate its tissue colonization [9, 11]. One such receptor type is proteoglycans (e.g. decorin and biglycan), which are com-

posed by a core protein and covalently linked glycan chains, as known as glycosaminoglycans (GAGs) [12-14]. Lyme borreliae less efficiently colonizes the tissues of mice defective in producing decorin or biglycan [15-16]. These spirochetes were also documented to bind to these proteoglycans and several GAGs, including heparan sulfate and dermatan sulfate [17-20], and the incubation of these molecules with Lyme borreliae inhibits spirochete attachment to cells [17, 20]. These results suggest that a spirochetes ability to bind to a proteoglycan or GAG mediates their attachment to host cells and tissues. Furthermore, *intravenous* inoculation of *B. burgdorferi* in conjunction with heparin, a GAG analog, reduces the vascular interaction of spirochetes [21-22]. This result supports the use of heparin to prevent hematogenous dissemination of Lyme borreliae.

In fact, heparin has been examined as antimicrobial therapeutic, since it is capable of modulating host immune responses against pathogens [23]. However, heparin has long been used as an anticoagulant to prevent clot formation, thus, this compound often triggers side effects such as thrombocytopenia and bleeding [24-25]. Additionally, the structure of unfractionated heparin is heterogenous and with great size of chains, which appears to be associated with this heparin's short half-life in humans (< 2 h) [26]. These properties increase the complexity of using heparin to prevent microbial infection. A low molecular weight fraction of heparin (< 8,000 Da) has a prolonged half-life [27-29]. We and others have further modified the structure of low molecular weight heparin to eliminate its anticoagulant activity [29-30]. This non-anticoagulant version of low molecular weight heparin (NACH) demonstrates no toxicity in mammalian hosts and has been used in humans for clinical trials [31-33]. The improved pharmacology and safety of NACH raises the possibility of testing the ability of this compound to prevent Lyme disease infection.

In this study, we synthesized NACH and gave it to mice prior to exposure to ticks carrying *B. burgdorferi* and examined the ability of NACH to prevent Lyme disease infection. We also investigated the mechanisms by which NACH blocked the infection onset and evaluated the possibility of using NACH a pre-exposure prophylaxis (PrEP) for Lyme disease.

RESULTS

NACH bound to low passage Lyme borreliae strains and prevented these spirochetes from attaching to proteoglycans and GAGs.

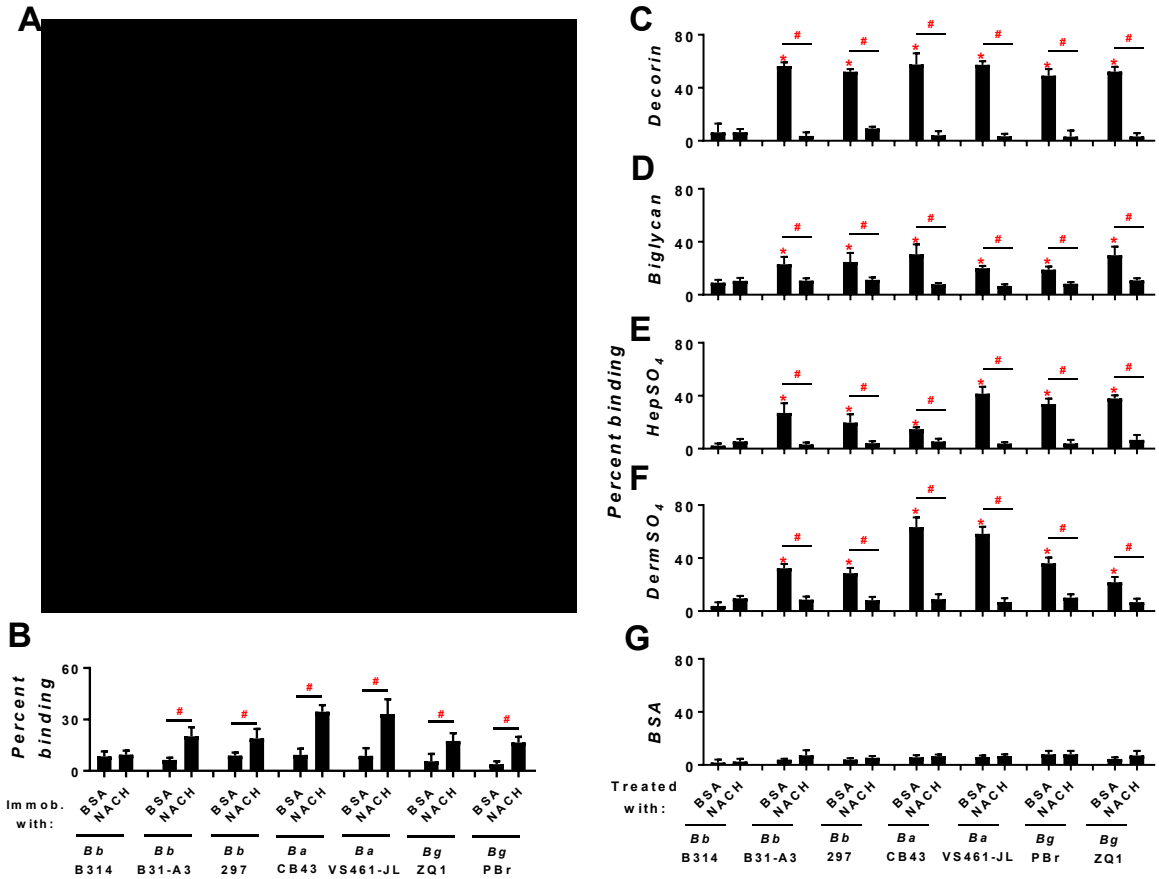

Figure 1. NACH bound to Lyme borreliae to prevent spirochete association with proteoglycans and GAGs. (A) Synthesis and structure of NACH: m + n = 16 for MW 12 kDa; R_1 = H or HSO_3; R_2 = Ac or HSO_3. **(B)** The percentage of *B. burgdorferi* strains B314, B31-A3, or 297, *B. afzelii* strains CB43 or VS461-JL, or *B. garinii* strains ZQ1 or PBr that bind to NACH or to BSA as a negative control, was determined by ELISA (see Experimental section). Each bar represents the mean of four independent determinations ± standard deviation. Significant differences in spirochetal binding between indicated wells determined using Mann-Whitney tests and are indicated (#, P < 0.05). **(C to G)** *B. burgdorferi* strains B314, B31-A3, or 297, *B. afzelii* strains CB43 or VS461-JL, or *B. garinii* strains ZQ1 or PBr was incubated with 2.5 mg ml^{-1} of NACH or BSA alone as a negative control for 1 h prior to be added to microtiter plate wells immobilized with **(C)** decorin, **(D)** biglycan, **(E)** heparan sulfate ($HepSO_4$), **(F)** dermatan sulfate (DermSO4), or **(G)** BSA (negative control). Each bar represents the mean of four independent determinations ± standard deviation. Significant reductions (P < 0.05) in spirochetal binding between different groups (#) or relative to BSA-treated spirochetes (*) are indicated using Mann-Whitney tests.

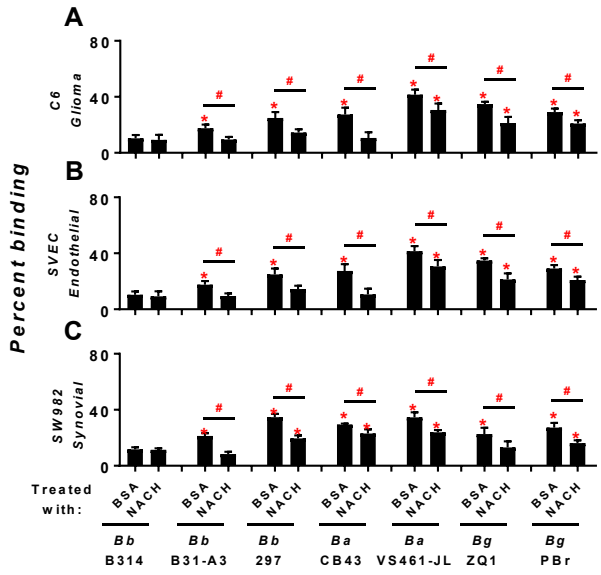

Figure 2. NACH bound to Lyme borreliae to block spirochetes' ability in attaching to mammalian cells. *B. burgdorferi* strains B314 (negative control), B31-A3, or 297, *B. afzelii* strains CB43 or VS461-JL, or *B. garinii* strains ZQ1 or PBr was incubated with 2.5 mg ml^{-1} of NACH or PBS alone as a negative control (none) for 1 h prior to be added to microtiter plate wells containing **(A)** C6 glioma, **(B)** SVEC endothelial, **(C)** SW982 synovial, or BSA (negative control, data not shown). Each bar represents the mean of four independent determinations ± standard deviation. Significant reductions ($P < 0.05$) in spirochetal binding relative to the strain B314 (*) or to BSA-treated spirochetes (#) are indicated using Mann-Whitney tests.

Commercially available porcine intestinal heparin was treated with nitrous acid for depolymerization, resulting in the low molecular weight heparin, dalteparin **(Figure 1A).** This dalteparin was then oxidized with sodium periodate followed by $NaBH_4$ reduction as described in our previous work **(Figure 1A)** [29-30]. The resulting product, NACH, was verified for its purity and low anticoagulant activity described in our recent study [29]. We next incubated the resulting NACH products or BSA (negative control) with different low passage and infectious Lyme borreliae species or strains, including *B. burgdorferi* strains B31-A3 and 297 (representing two distinct genotypes associated with human infection) [34], *B. afzelii* strains CB43 and VS461-JL, and *B. garinii* strains ZQ1 and PBr. A high passage, non-infectious, and non-adherent *B. burgdorferi* strain B314 was also included as negative control **(Table S1)**[35-36]. The resulting spirochetes were added to the NACH-coated microtiter plate wells for the determination of spirochete attachment. Less than ten percent of these Lyme borreliae strains bound to BSA as expected **(Figure 1B).** While the negative control strain B314 bound to NACH at levels no different from that to BSA, more than 15% of other low passaged strains were immobilized by NACH, at greater levels than that by BSA (**Figure 1B).** These results indicate invariable ability of spirochete strains and species to attach to NACH.

NACH is an analog of heparin, which is structurally similar to the GAG component of proteoglycans binding Lyme borreliae. This raises the possibility that NACH is capable of inhibiting spirochetes binding to these proteoglycans and GAGs. Thus, we mixed NACH or PBS (negative control) with the

Table 1. Serum concentration of NACH in mice 24 h after inoculation.

Treatment	Serum concentration of GAG (ng µl^{-1})[a]		
	$HepSO_4$[b]	$ChonSO_4$[c]	Hyaluronic Acid
NACH	828.8±1.06[d]	54417±1.19	561.6±1.18
PBS	712.1±1.03	51758±1.11	490.1±1.29

[a]All values represent the geometric mean ± geometric standard deviation of seven (for NACH-treated mice) or five (for PBS-treated mice) mice per group.
[b]Heparan sulfate
[c]Chondroitin sulfate
[d]Significant greater value than Heparan sulfate in the serum from PBS-treated mice using Mann-Whitney tests ($p = 0.0051$).

above-mentioned Lyme borreliae strains and added the mixture into the microtiter plate wells immobilized with different proteoglycans and GAGs. These ligands included decorin, biglycan, heparan sulfate, and dermatan sulfate, and BSA was included as control. As expected, the non-adherent strain B314 binds to these ligands at undetectable levels (lower than ten percent binding) **(Figure 1C to G).** All infectious spirochete strains when treated with PBS bind to decorin, biglycan, heparan sulfate, and dermatan sulfate but not BSA at the levels greater than the strain B314 **(Figure 1C to G).** However, after treated with NACH, these strains bind to the above-

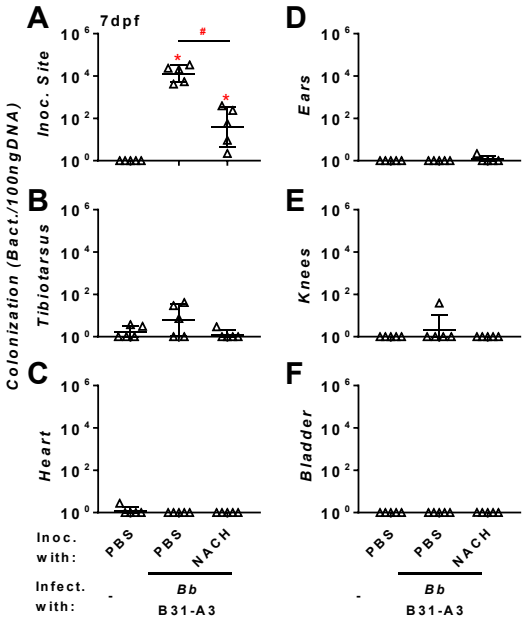

Figure 3. Spirochetes were eliminated at tick biting sites of skin in NACH-treated mice. Swiss-Webster mice were *subcutaneously* inoculated with NACH (5 mg per kg of mouse) or PBS at 24 h prior to be fed on by *I. scapularis* nymphs carrying *B. burgdorferi* strain B31. NACH (or PBS) was give daily at the same fashion until the mice were euthanized. At 7-days post tick feeding ("dpf"), mice were euthanized. The spirochete burdens in the **(A)** biting site of skin ("Inoc. Site"), **(B)** tibiotarsus joints ("tibiotarsus"), **(C)** heart, **(D)** ears, **(E)** knee joints ("knees"), and **(F)** bladder. Bacterial burdens were determined by qPCR and normalized to 100 ng total DNA. Uninfected, PBS-inoculated mice were included as control ("-"). Shown are the geometric mean ± geometric standard deviation of five mice per group in two experimental events. Significant differences ($p < 0.05$ by Kruskal-Wallis test with Dunn's multiple comparison) in the spirochete burdens relative to uninfected- and PBS-inoculated mice ("*") or between two treatment groups ("#") are indicated.

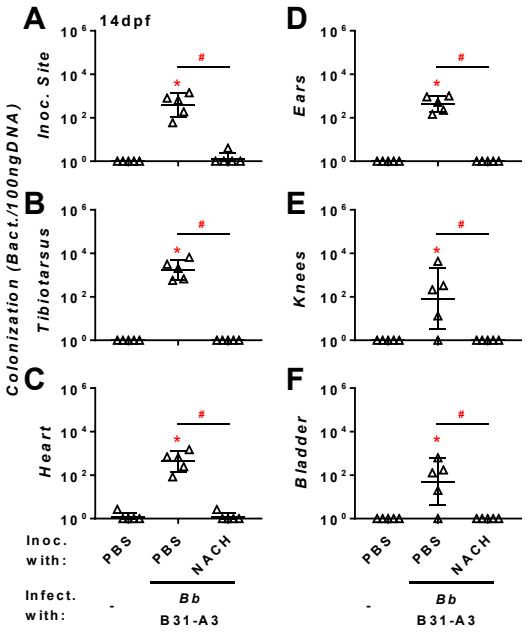

Figure 4. Spirochetes could not disseminate to distal tissues in NACH-treated mice. Swiss-Webster mice were *subcutaneously* inoculated with NACH (5 mg per kg of mouse) or PBS at 24 h prior to be fed on by *I. scapularis* nymphs carrying *B. burgdorferi* strain B31. NACH (or PBS) was give daily at the same fashion until the mice were euthanized. At 14-days post tick feeding ("dpf"), mice were euthanized. The spirochete burdens in the **(A)** biting site of skin ("Inoc. Site"), **(B)** tibiotarsus joints ("tibiotarsus"), **(C)** heart, **(D)** ears, **(E)** knee joints ("knees"), and **(F)** bladder. Ears were determined by qPCR and normalized to 100 ng total DNA. Uninfected, PBS-inoculated mice were included as control ("-"). Shown are the geometric mean ± geometric standard deviation of five mice per group in two experimental events. Significant differences ($p < 0.05$ by Kruskal-Wallis test with Dunn's multiple comparison) in the spirochete burdens relative to uninfected- and PBS-inoculated mice ("*") or between two treatment groups ("#").

mentioned proteoglycans or GAGs no better than the strain B314 **(Figure 1C to G)**, demonstrating that NACH has the ability to reduce spirochete attachment to proteoglycans and GAGs.

NACH inhibited spirochete adhesion to mammalian cells.

Since proteoglycans and GAGs are located on the surface of mammalian cells, we sought to determine the ability of NACH to block spirochete attachment to these cells. Different Lyme borreliae species or strains were thus incubated with NACH (or BSA, negative control) prior to being added to different mammalian cells types, including C6 glioma, SVEC endothelial, and SW982 joint synovial cells. These cell lines were selected as they were derived from the tissues that Lyme borreliae often colonize during infection. It is not surprising that less than ten percent of the high passage and non-adherent strain B314 attaches to these cell types **(Figure 2).** After treatment with BSA, the infectious *B. burgdorferi*, *B. afzelii*, and *B. garinii* strains all bound to C6, SVEC, and SW982 cells at the levels greater than the strain B314 **(Figure 2).** In contrast, treating these infectious Lyme borreliae strains with NACH resulted in lower levels of attachment to these cell lines, compared to the treatment of BSA **(Figure 2).** These findings show that NACH reduces Lyme borreliae attachment to mammalian cells.

NACH reduced spirochetes' ability to establish infection in mammalian hosts.

We next subcutaneously inoculated Swiss-Webster mice with NACH or PBS (negative control) to determine the ability of this compound to act as a PrEP in preventing Lyme disease infection **(Figure S1).** This mouse strain was used as it is outbred and thus more closely reflects the genetically variable background in humans. Additionally, this mouse strain has been commonly used as a model for mammalian Lyme disease infection [37-39]. Although comparable concentrations of chondroitin sulfate and hyaluronic acids were detected in NACH- and PBS-treated mice at 24 h post inoculation, we observed greater levels of heparan sulfate in the NACH-treated mice **(Table 1).** This result is in agreement with the fact of heparan sulfate has a similar disaccharide unit to NACH [29-30], and it also verifies the bioavailability of NACH through this inoculation route. Twenty-four-hours after administration, the *I. scapularis* nymphs carrying *B. burgdorferi* strains B31-A3 or 297 were allowed to feed on these mice till repletion **(Figure S1).** These mice were continuously inoculated daily at the same fashion with NACH until they were euthanized **(Figure S1).** The replete nymphs were weighed to evaluate their feeding quality, and the DNA extracted from nymphs and tissues at different time points were collected to determine their bacterial burdens.

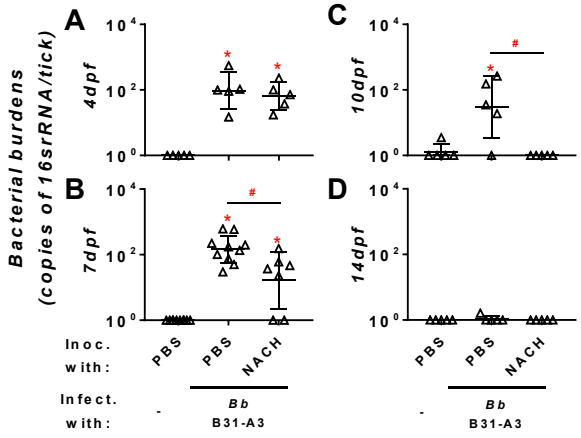

Figure 5. Spirochetes were cleared from the bloodstream of the NACH-treated mice at earlier onset than that of PBS-treated mice. Swiss-Webster mice were *subcutaneously* inoculated with NACH (5 mg per kg of mouse) or PBS at 24 h prior to be fed on by *I. scapularis* nymphs carrying *B. burgdorferi* strain B31. NACH (or PBS) was give daily at the same fashion until the mice were euthanized. The spirochete burdens in the bloodstream were determined by qPCR and normalized to 100 ng total DNA at **(A)** 4-, **(B)** 7-, **(C)** 10-, or **(D)** 14-days post tick feeding ("dpf"). Uninfected and PBS-inoculated mice were included as control ("-"). Shown are the geometric mean ± geometric standard deviation of seven (for 7dpf NACH-treated mice), ten (for 7dpf, PBS-treated mice) or five mice (for the other time points) per group in two experimental events. Significant differences ($p < 0.05$ by Kruskal-Wallis test with Dunn's multiple comparison) in the spirochete burdens relative to uninfected- and PBS-inoculated mice ("*") or between two treatment groups ("#") are indicated.

We found that the replete ticks feeding on NACH-inoculated mice do not weight differently from the ticks feeding on PBS-administrated mice. This trend applied to the ticks carrying strains either B31-A3 or 297 **(Figure S2A).** These results indicate that NACH does not interfere with tick feeding. Compared to flat nymphs, the spirochete burdens in fully engorged nymphs increased approximately ten-fold, as reported previously **(Figure S2B)** [40-41]. However, no differences in bacterial burdens were found in the strains B31- or 297-carrying nymphs feeding on NACH- or PBS-inoculated mice **(Figure S2B).** These observations show that NACH does not eliminate spirochetes in ticks during feeding.

We also found that no spirochetes are detectable at the biting sites in the skin of PBS-inoculated and uninfected mice at seven days post tick feeding, as expected **(Figure 3A).** At this time point, the bacterial burdens of the PBS- or NACH-administrated mice fed on by ticks carrying *B. burgdorferi* strain B31-A3 were greater than that in uninfected mice **(Figure 3A).** Between those infected mice, the spirochete loads were lower in NACH-treated individuals, compared to PBS-treated animals **(Figure 3A).** These results demonstrate the ability of NACH to reduce infection establishment by Lyme borreliae in mammalian hosts. We were unable to detect the spirochetes at heart, joints, ears, and bladder of PBS-inoculated and uninfected mice at seven days post tick feeding **(Figure 3B to F).** Similarly, the bacterial loads of either NACH- or PBS-treated animals were undetectable in these tissues, suggesting that *B. burgdorferi* has not disseminated to these distal tissues at seven days post tick feeding **(Figure 3B to F).**

NACH facilitated *B. burgdorferi* clearance at tick biting sites and bloodstream to prevent spirochete dissemination to distal tissues.

We sought to determine the burdens of *B. burgdorferi* strain B31-A3 in tick biting sites at 14 days post tick feeding. PBS-inoculated and uninfected mice did not develop detectable spirochete burdens at the biting site of skin **(Figure 4A).** Although we were able to detect bacteria at this tissue and this time point from the PBS-inoculated and the strain B31-A3-infected mice, no spirochetes were detected at the biting site of skin from NACH-inoculated mice **(Figure 4A).** Similarly, we also measured the spirochete burdens in tick biting sites of skin at 56 days post tick feeding and did not detect *B. burgdorferi* at this tissue of NACH-treated mice **(Figure S3A).** These results suggest that NACH inoculation promotes spirochete elimination at the infection-initiated site. Additionally, we were incapable of detecting spirochetes in the biting site of skin from NACH-administrated mice at 56 days after these mice were fed on by ticks carrying *B. burgdorferi* strain 297 **(Figure S3A).** This result suggests that such a NACH-mediated spirochete eradication at tick biting sites can be extended to other Lyme borreliae strains.

We also determined the burdens of *B. burgdorferi* strain B31-A3 in heart, joints, ears, and bladder at 14 days post tick feeding. While spirochetes were undetectable in these tissues of PBS-inoculated and uninfected mice, approximately 10^3 of

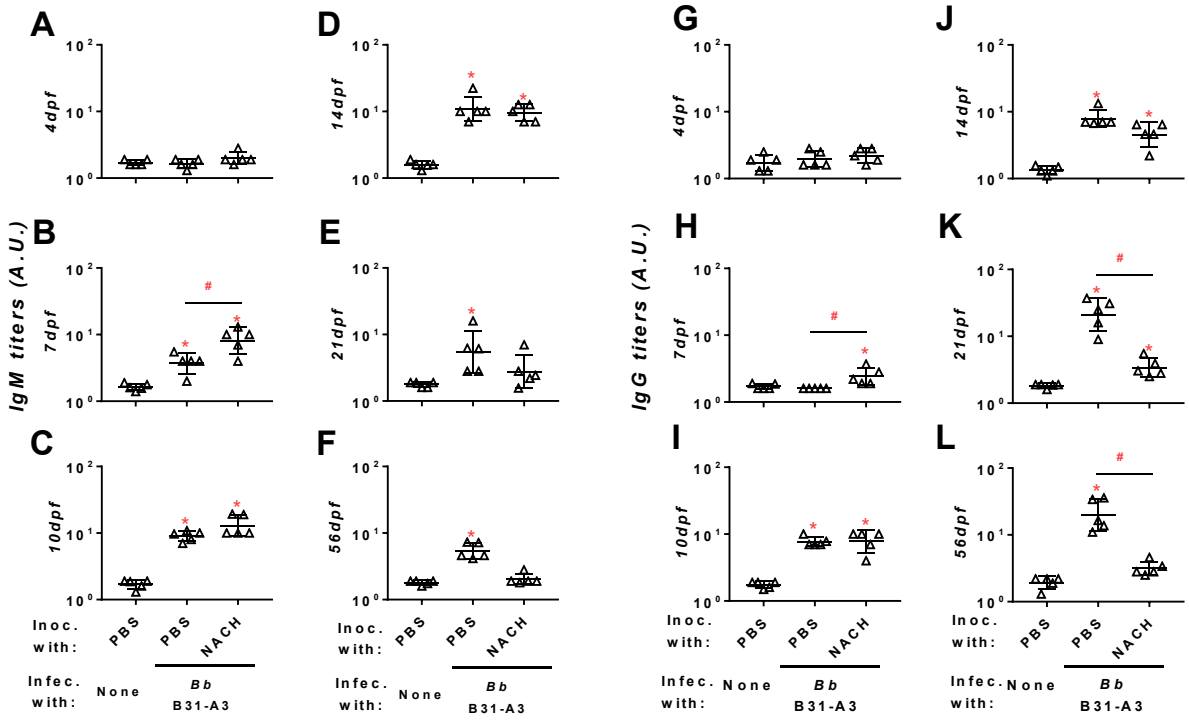

Figure 6. Mice administrated with NACH developed more robust antibodies against spirochetes than PBS-treated mice at early stages of infection. Swiss-Webster mice were *subcutaneously* inoculated with NACH (5mg per kg of mouse) or PBS at 24 h prior to be fed on by *I. scapularis* nymphs carrying *B. burgdorferi* strain B31-A3. NACH (or PBS) was give daily at the same fashion until the mice were euthanized. The sera were obtained at **(A and G)** 4-, **(B and H)** 7-, **(C and I)** 10-, or **(D and J)** 14-, **(E and K)** 21-, **(F and L)** 56-days post tick feeding ("dpf"). Uninfected and PBS-inoculated mice were included as control ("-"). The levels of IgG **(A to F)** and IgM **(G to L)** against *B. burgdorferi* strain B31-A3 were determined using quantitative ELISA as described in Section "Experimental section." Shown are the geometric mean ± geometric standard deviation of five mice per group in two experimental events. Significant differences ($p < 0.05$ by Kruskal-Wallis test with Dunn's multiple comparison) in the spirochete burdens relative to uninfected and PBS-inoculated mice ("*") or between two treatment groups ("#") are indicated.

the strain B31-A3 were found to colonize at heart, joints, ears, and bladder of mice administrated with PBS and fed on by ticks carrying this spirochete strain **(Figure 4B to F).** Interestingly, no bacteria were detected at these distal tissues of NACH-administrated mice followed by being infected using ticks carrying the strain B31-A3 **(Figure 4B to F).** Similar trends of undetectable colonization at joints, heart, ears, and bladder were found in NACH-treated mice at 56 days post feeding **(Figure S3B to F).** These observations suggest that NACH-treatment prevents spirochete dissemination to distal tissues. Further, we did not observe the colonization of any spirochetes at these tissues of NACH-treated mice when mice were infected with *B. burgdorferi* strain 297 **(Figure S3B to F).** These results clearly point out the ability of NACH to prevent multiple Lyme borreliae strains' dissemination.

The fact that Lyme borreliae dissemination requires the ability to survive in the bloodstream, raises the possibility that NACH may facilitate hematogenous clearance of spirochetes, preventing dissemination. Thus, we determined the spirochete burdens in the mouse bloodstream at different time points after tick feeding. *B. burgdorferi* strain B31-A3 survived at undistinguishable levels in the bloodstream of PBS- or NACH-treated mice at 4 days post tick feeding **(Figure 5A).** However, compared to PBS-treated mice where the strain B31-A3 survival was close to uninfluenced, the burdens of this strain in NACH-treated mice were reduced at seven days and undetectable at 10 days post feeding **(Figure 5B and C).** Fourteen days after tick feeding, we were unable to detect spirochetes in the bloodstream of either PBS-inoculated or NACH-treated mice **(Figure 5D)**. This result is in agreement with the adaptive immune clearance of Lyme borreliae in the bloodstream at this time point [42-43]. Taken together, these findings indicate the faster kinetics of hematogenous clearance of *B. burgdorferi* in NACH-treated mice.

NACH-treated mice developed more robust early antibody immune responses against spirochetes after Lyme disease infection.

Our finding that *B. burgdorferi* is more rapidly eliminated in NACH-treated mice than that in PBS-inoculated mice, leads to the possibility that NACH facilitates spirochete clearance. However, we found that the number of motile *B. burgdorferi* strains B31-A3 or 297 does not decrease after treatment of NACH, similar to these strains treated with PBS (data not shown). Additionally, both strains when treated with NACH had doubling times (generation time) no different than them treated with PBS **(Table S2).** These results indicate that NACH does not directly kill Lyme borreliae. As antibody responses play a key role to clear Lyme borreliae during infection, we next sought to examine whether such immune responses are modulated by NACH. Mice were given NACH (or PBS) one day prior to the exposure to ticks carrying *B. burgdorferi* strain B31-A3 and continuously administrated with NACH daily after infection. Blood was collected at different time points to examine for the levels of IgG and IgM against spirochetes. As expected, IgM titers in infected mice treated with either NACH or PBS were no greater than that in uninfected mice at 4 days post tick feeding **(Figure 6A).** The mice fed on by nymphs under either treatment developed greater titers of IgM than the uninfected mice at 7, 10, and 14 days post tick feeding, indicating that both mice were infected by *B. burgdorferi* **(Figure 6B to D).** However, while the IgM levels of PBS-treated and *B. burgdorferi*-infected mice remained higher than that in uninfected mice at 21- and 56-days post feeding, that antibody levels of infected mice treated with NACH were no different from that in uninfected mice **(Figure 6E and F).** These results are consistent with the fact that spirochetes are cleared from tissues and bloodstream at those time points in NACH-treated mice **(Figure 4, 5, and S3).** Interestingly, after infection, NACH-treated mice developed greater IgM titers than PBS-inoculated mice at 7 days post tick feeding. This result suggests that NACH triggers efficient IgM responses at early stages of infection **(Figure 6B).**

Similarly, all the mouse groups did not generate detectable titers of IgG at 4 days post feeding **(Figure 6G).** The infected mice under either NACH or PBS treatment developed greater IgG titers than the uninfected mice at 10, 14, and 21 days post feeding **(Figure 6I to K).** The IgG titers of NACH-treated, *B. burgdorferi*-infected mice were lower than that in PBS-inoculated, spirochete-infected mice at 21 days post tick feeding and undetectable at 56 days post tick feeding **(Figure 6K and L).** These data are in agreement with the elimination of *B. burgdorferi* in NACH-treated mice at these time points. Similar to IgM, the IgG titers in infected mice treated with NACH were greater than that in the infected mice treated with PBS at 7 days post tick feeding **(Figure 6H)**. These results suggest the ability of NACH to induce early IgG responses. Taken together, these observations indicate that NACH administration triggers robust antibody immune responses against spirochetes early during Lyme disease infection.

DISCUSSION

Lyme borreliae bind to GAGs and proteoglycans [17-20], and such binding activity facilitates spirochete attachment to mammalian cells/tissues under static [35, 44-47], or shear stressed conditions [20-22, 48-50]. Among those ligands, decorin-, biglycan-, and dermatan sulfate were reported to bind to spirochetes, promoting tissue colonization of spirochetes and Lyme disease-associated manifestations [15-16, 51-53]. We found that Lyme borreliae species and strains display universal capability of binding to NACH, an analog of GAGs. We also demonstrated that NACH-treated spirochetes display reduced binding ability to different purified GAGs and proteoglycans, compared to untreated bacteria. Our findings raise a possibility that NACH blocks spirochete binding to GAGs and proteoglycans, inhibiting the colonization and disease manifestations caused by Lyme borreliae infection. This notion is supported by our observation of less efficient spirochete colonization at inoculation sites and distal tissues in the mice treated with NACH, compared to negative control mice. Further, such GAG- and proteoglycan-binding activities of Lyme borreliae have been attributed to several GAG- and/or proteoglycan-binding proteins on the surface of spirochetes [44-45, 51, 54-56]. Additionally, spirochetes deficient of some of these GAG- and proteoglycan-binding outer surface proteins less efficiently colonize at tissue and trigger manifestations than the parental wild type strain [47, 52-53]. Thus, our finding of NACH's efficacy to prevent

spirochete colonization leads to an intriguing question: Does NACH target these Lyme borreliae outer surface proteins to inhibit dissemination? Such a question warrants further investigations.

Vertebrate animals develop innate immune responses to eradicate Lyme borreliae immediately after spirochetes invade these hosts, which serve as a bottleneck prior to infection establishment [57-61]. Adaptive immune responses are activated later, which control spirochete burdens, resulting in reduced and persistent colonization at tissues [62-65]. Specifically, the levels of antibodies against Lyme borreliae or their outer surface antigens in sera have been correlated with the spirochete loads at tissues, supporting the role of B cell responses in mediating Lyme borreliae clearance [66-67]. Therefore, a more robust antibody production at earlier infection onset would be expected to facilitate more efficient clearance of spirochetes. Such effective eradication could then prevent spirochete dissemination to and colonization at distal tissues. In fact, we found that mice inoculated with NACH followed by being infected with Lyme borreliae develop greater levels of IgG and IgM against spirochetes at early stages of infection. These results are supported by the previous findings showing low molecular weight heparin is capable of modulating host immune responses, resulting in more efficient elimination of pathogenic bacteria by mammalian hosts [23]. Additionally, the kinetics of NACH-mediated robust antibody production match the onset of spirochete eradications at tick biting sites and bloodstream. This coincidence suggests that high levels of antibody generation against spirochetes at early stages of infection is likely one of the mechanisms for NACH to prevent Lyme disease infection.

In this study, we could not detect spirochetes at 56 days post tick feeding when mice were given NACH prior to the exposure to ticks carrying Lyme borreliae and introduced daily after infection. Six different tissues previously shown to be colonized by Lyme borreliae after tick feeding were included. These results do not rule out the possibility that spirochetes remain colonizing other untested tissues. However, that scenario is unlikely as the fact of the decreasing trends of antibodies against spirochetes from 14 to 21 days after tick feeding and the seronegative results at 56 days post tick feeding in NACH-treated mice. Furthermore, antibiotics are available as prophylaxis to prevent Lyme disease immediately after tick exposure[68]. However, the fact that many people do not notice a tick bite addresses the need of the prevention prior to tick infection. The only currently developed PrEP is a monoclonal antibody, which blocks tick-to-host transmission of Lyme borreliae by targeting a spirochete outer surface protein, OspA [69]. Unlike that prophylaxis, NACH targets GAG/proteoglycan-binding activity and upregulates antibody immune responses, which are not Lyme borreliae-specific. Thus, NACH may be potentially extended to prevent the infection caused by other pathogens.

CONCLUSION

Our results highlight the potential of using NACH as PrEP to prevent Lyme disease infection, but it is noted that the subcutaneous route and a comparatively short half-life of NACH regimen used in this study (~12 h) may not be ideal for PrEP development [33]. Many excipients have been used to formulate heparin to enhance the oral bioavailability and increase the half-life [70-74]. These findings shed a light for further developing NACH as a more suitable compound for human use as Lyme disease PrEP. Additionally, oral administration of prevention by targeting the reservoir animals has been examined as a strategy to reduce the risk of exposure to Lyme borreliae [75-78]. Thus, our findings also provide a possibility to use NACH as a bait for reservoir hosts in Lyme disease prevention through the formulation to enhance oral bioavailability and half-life. In this proof-of-concept study, we examined the possibility of using NACH prior to tickborne Lyme borreliae transmission to prevent spirochete colonization. We also investigated the potential mechanisms that drive NACH-mediated protection against Lyme disease. The results derived from this study will provide the foundation to ultimately enable us to reduce the burdens of human Lyme Disease.

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 was approved by the Institutional Animal Care and Use Committee of Wadsworth Center, New York State Department of Health (protocol Docket Number 19-451). All efforts were made to minimize animal suffering.

Mouse, ticks, bacterial strains, antisera, and enzymes.

Swiss-Webster mice were purchased from Taconic (Hudson, NY). *Ixodes scapularis* tick larvae were obtained from BEI Resources (Manassas, VA). *B. burgdorferi*-infected nymphs were generated as described in the section "Mouse infection experiments by ticks." All *B. burgdorferi*, *B. afzelii*, and *B. garinii* strains used in this study were grown in BSK-II completed medium **(Table S1).** To generate the antisera against different Lyme borreliae species, *B. burgdorferi* strain B31-A3, *B. afzelii* strain VS461-JL, or *B. garinii* strain ZQ1 were *subcutaneously* introduced into mice (10^6 bacteria per mouse). At 21 days post infection, ear biopsy (one biopsy per mouse) was collected to determine the infectivity of the particular strain used to infect that mouse using quantitative PCR (qPCR; see section "Quantitative PCR" and previous description [53]). Sera were collected from the qPCR-positive mice and verified its seropositivity to the particular strain used to infect that mouse using ELISA as described previously [79]. Chondroitin lyase ABC from *Proteus vulgaris* was expressed in in our laboratory. Recombinant *Flavobacterial* heparin lyases I, II, and III were expressed in our laboratory previously [80].

The production, purification, and verification of NACH.

NACH was synthesized from the low molecular weight heparin, dalteparin through nitrous acid depolymerization of porcine intestinal heparin), followed by periodate oxidation as described in our previous work [29-30]. Briefly, one gram of dalteparin (>95% purity) in aqueous solution (8.75 mL, pH 5.0) was added to freshly prepared 140 mM $NaIO_4$ solution (25 mL, pH 5.0) in a single portion with stirring. The oxidation was carried out at 4 °C for 24 h in the dark before the solution was desalted using 3 kDa cutoff spin columns. NaBH4 (50 mg) was added to the reaction to reduce the generated aldehydes. NACH was precipitated by adding ethanol to 80% (v/v). The final NACH product (>95% purity) was obtained by desalting and lyophilization. The structure of NACH was confirmed by NMR with the average MWs (~3870 Da) measured by gel permeation chromatography (GPC). Very low anticoagulant activity (6 U/mg as anti-Xa potency) of NACH was detected by using BIOPHEN heparin anti-Xa (2 stages) kit (Aniara Diagnostica, West Chester, OH). GPC and disaccharide analysis confirmed the >95% purity of NACH using USP enoxaparin was used as a standard.

Determining the serum concentration of GAGs derived from NACH.

Mice were *subcutaneously* injected with NACH (5 mg per kg of mouse), and sera were collected from these mice at 24 hours post inoculation. The determination of GAG composition in the sera has been described previously [81-82]. Basically, twenty µl of sera was loaded onto a spin column (Molecular molecular weight cut-off, 3 kDa)[76] and then the column was washed with distilled water. The upper solution was mixed with 300 µl of digestion buffer[76] and the mixture of GAG lyases, including heparin lyase I, II, and III as well as chondroitin lyase ABC (10 mU for each GAG lyase). After incubated at 37 °C for overnight, the reaction mixture was applied to a spin column with molecular weight cutoff as 3 KDa to terminate the reaction by GAG lyases. After air-drying the flow through, the sample was labeled with 2-aminoacridone (AMAC) by being incubated with 10 µl of AMAC (0.1 M, Sigma-Aldrich) in DMSO/acetic acid (17/3,V/V, Sigma-Aldrich) at room temperature for 10 min, followed by being mixed with 10 µl of $NaBH_3CN$ (1 M) at 45 °C for 1 h. The resulting samples were spun down, and the supernatant was collected and stored in a light resistant container at room temperature until analyzed via LC-MS/MS.

LC was performed on an Agilent 1200 LC system at 45 °C using an Agilent Poroshell 120 ECC18 (2.7 µm, 3.0 × 50 mm) column (Agilent Technology, Santa Clara, CA). The mobile phase A (MPA) and B (MPB) were ammonium acetate (50mM) and methanol, respectively. The gradient used for purification was 5 to 45% of MPB/95 to 55% MPA from 0 to 10 minutes, 45 to 100% of MPB/55% to 0% of MPA from 10 to 10.2 minutes, 100% of MPB/0% of MPA from 10.2 to 14 minutes, 100 to 5% of MPB/0 to 95% of MPA from 14 to 22 minutes. The flow rate was 300 µl per minute. A triple quadrupole mass spectrometry system equipped with an ESI source (Thermo Fisher Scientific, San Jose, CA) was used as a detector. The online MS analysis was at the Multiple Reaction Monitoring (MRM) mode. The parameters for MS analysis are as follows: negative ionization mode with a spray voltage of 3000 V, a vaporizer temperature of 300 °C, and a capillary temperature of 270 °C.

Binding of spirochetes to NACH.

One hundred µl of NACH or BSA (negative control) at the concentration of 1 mg ml^{-1} in the coating buffer (0.1 M carbonate/bicarbonate, pH 9.6 [51]) were added to ELISA microtiter plate wells (ThermoScientific, Pittsburgh, PA). The plates were then incubated for 16 h at 4°C followed by being blocked using 5% PBS-BSA. Spirochetes suspended in BSK-H medium were added to wells with the immobilized NACH at 1×10^6 spirochetes per well. To enhance spirochete cell contact, the plates were centrifuged at 106 g for 5 min and then rocked at room temperature for 1 h. Unbound bacteria were removed by washing with PBS containing 0.2% BSA. To generate the plate wells with bacteria for the purpose of normalization, spirochetes (1×10^6 per well) suspended in the coating buffer were added to plate wells. Immobilized Lyme borreliae or the spirochetes bound on NACH (or BSA) were fixed using 3% of paraformaldehyde followed by 100% chilled methanol. After the plates were air-dried, the antisera against *B. burgdorferi*, *B. afzelii*, or *B. garinii* (1:200x) and HRP-conjugated goat anti-mouse IgG (ThermoFisher; 1:2,000x) were used as primary and secondary antibodies, respectively. The plates were washed three times with PBST (0.05% Tween 20 in PBS), and 100µL of tetramethyl benzidine solution (ThermoFisher) was added to each well and incubated for five minutes. The reaction was stopped by adding 100µL of 0.5% hydrosulfuric acid to each well. The absorption at 405nm of each well was obtained by reading the plates at that wavelength using a Tecan Sunrise Microplate reader (Tecan, Morrisville NC). The percent binding of spirochetes was derived from the absorption values of spirochete attachment to each well normalized to the values obtained from the wells immobilized with 1×10^6 of those spirochetes.

Inhibition of proteoglycan, GAG, mammalian cell binding with exogenous NACH.

One hundred microliters of human decorin (gifts from Drs. David Mann and Nancy Ulbrandt), human biglycan (Sigma-Aldrich, St. Louis, MO), heparin sulfate (Sigma-Aldrich), porcine dermatan sulfate (Millipore Billerica, MA), or BSA (negative control) at the concentration of 1 mg ml^{-1} in the coating buffer were added to ELISA microtiter plate wells. The plates were then incubated for 16 h at 4°C followed by being blocked using 5% PBS-BSA. For the plate wells cultivated with mammalian cells, C6 rat glioma, SVEC rat endothelial, or SW982 human synovial cells (gifts provided by Dr. John Leong) were added into microtiter plate wells (1×10^5 per well). Spirochetes (1×10^6) were prepared as described in the section "Binding of spirochetes to NACH" and incubated for 30 min at room temperature in BSK-H supplemented with 1 mg ml^{-1} of NACH. Spirochetes were then added into these proteoglycans- or GAGs-coated plate wells and incubated at room temperature for one hour. The percent binding of spirochetes was determined as described earlier.

Determination of spirochete killing activity of NACH *in vitro*.

We evaluated the viability of NACH-treated spirochetes with a survival assay and growth curve. To determine spirochetes' viability, 10^6 *B. burgdorferi* strains B31-A3 or 297 in BSK-II complete were treated with PBS buffer or 100ug NACH in this buffer and incubated at 33°C in triplicate. The motility of bacteria as determined as viable cells were observed initially and again after 24 hours using dark field microscopy by evaluating four fields of view[83]. The percentage of motile spirochetes was determined as described [40, 83] These spirochetes were then seeded in BSK II medium, also in triplicate, to determine the spirochetes' generation time as previously described [84].

Generation of nymphal ticks carrying *B. burgdorferi*.

The experimental procedure has been described previously [40]. Basically, four-week-old male and female C3 deficient Balb/c mice were infected with 10^5 of *B. burgdorferi* strain B31-A3 or 297 by subcutaneous injection (1×10^5 spirochetes per mouse). The plasmid profiles of strain B31-A3 were verified prior to infection as described to ensure no loss of plasmids[85-87]. The strain 297 was maintained at passages less than ten. The ear punches from those mice were collected and examined the presence of spirochete DNA at 7 days post infection using qPCR as described to confirm the infection of these mice [40]. At 14 days post infection, the uninfected *I. scapularis* larvae were allowed to feed to repletion on those *B. burgdorferi*-infected mice as described previously [40]. Approximately 100 to 200 larvae were allowed to feed on each mouse. The engorged larvae were collected and allowed to molt into nymphs in 4 to 6 weeks in a desiccator at room temperature and 95% relative humidity in a room with light dark control (light to dark, 12: 12 hours).

NACH inoculation and *B. burgdorferi* infection via ticks.

Four-week-old female Swiss-Webster mice were *subcutaneously* inoculated with NACH in PBS (5mg per kg of mouse) or PBS (control). The tick infection procedure was described previously [40]. Basically, at 24 h post inoculation, ten nymphs were allowed to feed on each mouse till repletion, and replete nymphs were collected for weight measurement. After placing nymphs on those mice, these mice were continuously given NACH or PBS daily in the same fashion. Uninfected and PBS-inoculated mice were included as negative control. Blood were collected at 4, 7, 10, and 14 days post tick feeding whereas sera were obtained at 4, 7, 10, 14, 21, and 56 days post tick feeding. Mice were euthanized at 7, 14, and 56 days after tick feeding, and the feeding site of the skin, the tibiotarsus and knee joints, heart, ears, and bladder were collected. Animal tissues, blood, and ticks were used to quantitatively evaluate the levels of colonization during infection (see section "Quantitative PCR") while sera were used to measure the levels of antibodies (see section "Quantification of antibody titers against *B. burgdorferi*").

Quantitative PCR.

DNA was extracted using EZ-10 Spin Column Blood DNA Mini-Prep Kit (BioBasic, Inc., Markham, Ontario, Canada). The quantity and quality of DNA for each tissue, tick, or blood sample were assessed by measuring the concentration of DNA and the ratio of the UV absorption at 260 to 280 using a Nanodrop 1000 UV/Vis spectrophotometer (ThermoFisher, Waltham, MA). The 280:260 ratio was between 1.75 to 1.85, indicating the lack of contaminating RNA or proteins. qPCR was performed to quantify spirochete loads through amplification of the *16s rRNA* gene **(Table S3)**[88]. The reactions were performed with the instrument and reagent as described[83]. Cycling parameters for the 16srRNA gene were 50°C for 2 minutes, 95°C for 10 minutes, and 45 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Each biological replicate was performed in duplicate due to the potential variation of these runs. The number of *16s rRNA* copies was calculated by fitting the establishing a threshold cycle (Cq) standard curve of a known number of the *16s rRNA* gene extracted from cultivated respective spirochete strains. Five samples from each tissue were also applied to qPCR using mouse nidogen primers to assure that low signals of the results are not due to the presence of PCR inhibitors **(Table S3)**[53, 89]. 10^7 copies of the mouse nidogen gene from 100ng of each DNA sample were detected as predicted, ruling out the presence of PCR inhibitors in these samples.

Quantification of antibody titers against *B. burgdorferi*.

The measurement of IgG and IgM titers against spirochete strains was performed using ELISA. In brief, microtiter plate wells were coated with *B. burgdorferi* strains B31 or 297 (1×10^6 spirochetes per well). After blocking with 5% PBS-BSA, mouse serum diluted in 50 µl of PBS (1:100x, 1:300x, or 1:900x) was added to each well. The antibodies and the reading protocol of the equipment have been described [83]. We determined the titers by obtaining the greatest maximum slope of optical density/minute per sample. The values of such maximum slopes were multiplied by the respective serum dilution factor as shown as arbitrary units.

Statistical analysis.

Significant differences between samples were determined using the Kruskal-Wallis test with Dunn's multiple comparison, or the Mann-Whitney test. A p-value < 0.05 was considered to be significant.

SUPPORTING INFORMATION

Experimental timeline of NACH inoculation and tick infection to mice **(Figure S1);** Replete ticks acquiring blood from NACHs or PBS-treated mice display similar weight and spirochete burdens **(Figure S2);** Spirochetes were undetectable at 56 days post tick feeding of NACH-treated mice **(Figure S3)**; Strains used in this study **(Table S1)**; The generation time of PBS- and NACH-treated *B. burgdorferi* strains used in this study **(Table S2)**; Primers used in this study **(Table S3)**.

AUTHOR INFORMATION

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CONFLICT OF INTEREST

The authors declare no competing financial interest.

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ABBREVIATIONS

LD; Lyme disease

PrEP: Pre-exposure prophylaxis

PG; Proteoglycan

GAG; Glycosaminoglycan

NACH; Non-anticagulant heparin

*Bb*sl; *Borrelia burgdorferi* sensu lato

B. burgdorferi; *B. burgdorferi* sensu stricto

$HepSO_4$; Heparan sulfate

$ChonSO_4$; Chondroitin sulfate

$DermSO_4$; Dermatan sulfate

Bb; *B. burgdorferi*

Ba; *B. arzelii*

Bg; *B. garinii*

dpf; Days post tick feeding

MRM; Multiple Reaction Monitoring

TMB; Tetramethyl benzidine

AU.; Arbitrary unit

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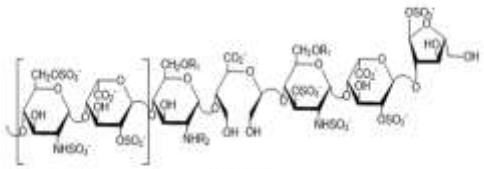

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