



Antibody Response to Lyme Disease Spirochetes in the Context of VlsE-Mediated Immune Evasion

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ABSTRACT Lyme disease (LD), the most prevalent tick-borne illness in North America, is caused by *Borrelia burgdorferi*. The long-term survival of *B. burgdorferi* spirochetes in the mammalian host is achieved through VlsE-mediated antigenic variation. It is mathematically predicted that a highly variable surface antigen prolongs bacterial infection sufficiently to exhaust the immune response directed toward invariant surface antigens. If the prediction is correct, it is expected that the antibody response to *B. burgdorferi* invariant antigens will become nonprotective as *B. burgdorferi* infection progresses. To test this assumption, changes in the protective efficacy of the immune response to *B. burgdorferi* surface antigens were monitored via a superinfection model over the course of 70 days. *B. burgdorferi*-infected mice were subjected to secondary challenge by heterologous *B. burgdorferi* at different time points postinfection (p.i.). When the infected mice were superinfected with a VlsE-deficient clone (Δ VlsE) at day 28 p.i., the active anti-*B. burgdorferi* immune response did not prevent Δ VlsE-induced spirochetemia. In contrast, most mice blocked culture-detectable spirochetemia induced by wild-type *B. burgdorferi* (WT), indicating that VlsE was likely the primary target of the antibody response. As the *B. burgdorferi* infection further progressed, however, reversed outcomes were observed. At day 70 p.i. the host immune response to non-VlsE antigens became sufficiently potent to clear spirochetemia induced by Δ VlsE and yet failed to prevent WT-induced spirochetemia. To test if any significant changes in the anti-*B. burgdorferi* antibody repertoire accounted for the observed outcomes, global profiles of antibody specificities were determined. However, comparison of mimotopes revealed no major difference between day 28 and day 70 antibody repertoires.

KEYWORDS *Borrelia burgdorferi*, Lyme disease, antibody response, suppression, protective efficacy, VlsE, antibody repertoire, mimotopes

Lyme disease (LD), the most prevalent tick-borne illness in North America and Europe, is caused by spirochetes in the genus *Borrelia*. *Borrelia burgdorferi*, the principal human pathogen in the United States, is responsible for approximately 300,000 LD cases per year (1). LD is problematic because early diagnosis is easily missed due to flu-like symptoms, which only transiently appear in humans during an early stage of disease (2–5). When missed and therefore left untreated, LD becomes chronic, presenting itself as skin lesions, arthritis, and carditis and occasionally with subsequent nervous system involvement (6, 7). No preventable or therapeutic vaccine for humans is currently available.

The long-term survival of *B. burgdorferi* spirochetes in the mammalian host is achieved through the *B. burgdorferi* antigenic variation system (8). This elaborate system, first identified on a 28-kb linear plasmid (lp28-1) of the *B. burgdorferi* B31 strain, is

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composed of a *vlsE* expression site and 15 noncoding silent cassettes. As a result of segmental conversion from the cassettes into the *vlsE* gene, variants of the VlsE (variable major protein-like sequence expressed) surface lipoprotein are generated (9, 10). The *vls*-mediated variation of VlsE is absolutely required for persistence in mice as murine antibody clears the *vls*-deficient *B. burgdorferi* clone or the *B. burgdorferi* clone with nonswitchable VlsE (sVlsE, for static VlsE) (11–17). Besides VlsE, however, *B. burgdorferi* expresses numerous other surface (lipo)proteins that, in contrast to VlsE, are invariant (18). Antibody developed to non-VlsE surface antigens can protect mice from *B. burgdorferi* infection when variable VlsE is absent (17).

Two potential, not mutually exclusive, mechanisms of *vls*-mediated avoidance have been proposed (13, 17, 19–21). The first is *vls*-mediated masking whereby VlsE may physically shield *B. burgdorferi* surface antigens from antibody. The second is VlsE-mediated immune suppression (17, 22). As an immunodominant surface lipoprotein (23), VlsE may be directly or indirectly involved in suppression of the host antibody response. A mathematical model that considers the interplay between bacterial pathogens with an antigenic variation system, the immune response, and immune exhaustion may support the latter (24). Specifically, the model predicted that antigenic variation of dominant antigens of *Trypanosoma* or *Plasmodium falciparum* prolongs infection sufficiently to allow the immune response against invariant antigens to become exhausted. This exhaustion was predicted to occur before the immune response to invariant antigens could control infection. It is therefore plausible that, during *B. burgdorferi* infection, the antibody response to invariant (non-VlsE) surface antigens is suppressed via direct or indirect involvement of highly variable VlsE proteins. In this case, it is expected that antibody to invariant surface antigens will become inefficient in clearing *vls*-deficient *B. burgdorferi* clones. The central hypothesis that this study attempts to test states that the protective efficacy of the antibody response to *B. burgdorferi* non-VlsE surface antigens declines as *B. burgdorferi* infection progresses.

A recently developed superinfection model (25) was utilized to assess whether the protective efficacy of the host antibody changes over the course of *B. burgdorferi* infection. Furthermore, an approach involving random peptide phage display libraries (RPPDL) and next-generation sequencing (NGS) was undertaken to compare specificities of serum antibody developed during the early and late stages of *B. burgdorferi* infection. Overall, the present data show that the protective efficacy of the antibody response to VlsE and other surface antigens does change as *B. burgdorferi* infection progresses in the murine host.

RESULTS

Generation and characterization of the Δ VlsE Gent^r clone. In order to test whether the protective efficacy of host antibody to non-VlsE surface antigens declines as the *B. burgdorferi* infection progresses, a recently developed superinfection model was utilized (25). The experimental design involved wild-type *B. burgdorferi* 297 strain (26) and B31 A3 clones with antibiotic resistance cassettes for the primary and secondary challenge (superinfection), respectively. The antibiotic resistance allowed us to differentiate between the primary and superinfecting *B. burgdorferi* clones. Previously generated B31 A3 lp25::kan (WT Kan^r) and B31 A3 lp28-1 Δ vls (Δ VlsE Kan^r) clones were initially chosen for the *in vivo* assay (16, 25). However, the use of the Δ VlsE Kan^r clone in the superinfection model represented a potential caveat. In the prior work, it was noticed that a truncated lp28-1 plasmid was lost by *B. burgdorferi* spirochetes upon their recovery from infected C3H or SCID mice (17). To overcome this, a *vls*-deficient mutant that would retain an antibiotic resistance cassette *in vivo* had to be generated. This was achieved by an insertion, via allelic exchange, of a gentamicin (*gent*) antibiotic resistance cassette in the *bbe02* locus of lp25. The lp25 plasmid is essential for murine infectivity (12, 27), whereas inactivation of *bbe02*, a putative restriction modification gene, does not result in loss of infectivity in mice (28–30). Thus, B31 A3 lp28-1 Δ vls lp25::gent (Δ VlsE Gent^r), the clone that possessed both the kanamycin (*kan*) and

TABLE 1 Spirochetemia of superinfecting *Borrelia burgdorferi* detected in 297-infected C3H mice at day 7 post-secondary challenge

Day of superinfection post-primary infection	Frequency of superinfecting clones detected in blood ^a			
	WT Kan ^r	Δ VlsE Kan ^r	Δ VlsE Gent ^r	Δ VlsE Kan ^r + Δ VlsE Gent ^r
14	3/5	4/5	3/5	7/10
21	0/5	1/5	3/5	4/10
28	1/5	5/5	5/5	10/10
42	1/5	3/5	2/5	5/10
56	2/5	0/5	0/5	0/10
70	5/5	2/5	0/5	2/10

^aValues listed correspond to numbers of cultures positive/number of cultures tested.

gentamicin resistance cassettes on Ip28-1 and Ip25, respectively, was generated. A total of 20 transformants were chosen for initial PCR analysis to screen for the *kan* gene. Five clones were further PCR tested for the presence of all parental *B. burgdorferi* plasmids. Infectivity of a Δ VlsE Gent^r clone that retained the full parent plasmid profile was verified. The Δ VlsE Gent^r clone demonstrated spirochetemia in 100% of C3SnSmn.CB17-*Prkd^{scid}/J* (SCID) and C3H/HeNHsd (C3H) mice (five animals per group) (see Table S1 in the supplemental material). As expected, the Δ VlsE Gent^r clone was not able to establish persistent infection in C3H mice due to the lack of the *vls* locus (16).

Each superinfecting *B. burgdorferi* clone was used as a host-adapted variant. Host adaptation allowed *B. burgdorferi* to presumably mimic expression of surface antigens at levels comparable to those found during active infection at the time of challenge. For example, VlsE expression becomes approximately 32-fold higher in mice than that detected under *in vitro* growth conditions (23). To obtain host-adapted *B. burgdorferi* clones, the *in vitro*-grown WT Kan^r, Δ VlsE Kan^r, or Δ VlsE Gent^r clone was subcutaneously injected into SCID mice as previously described (17, 31). Ears from the infected SCID mice were then harvested at day 21 postinfection (p.i.). The infectivity of host-adapted WT Kan^r, Δ VlsE Kan^r, or Δ VlsE Gent^r was every time confirmed in C3H mice at the time of or a few days after each superinfection (three mice per group) (Tables S2, S3, and S4). Blood (~50 μ l) and other murine tissues (bladder, heart, ear, and joint) were harvested at days 7 and 21 postchallenge, respectively, from each control mouse. The murine tissues were incubated in Barbour-Stoenner-Kelly II (BSK-II) medium at 35°C under 2.5% CO₂ for up to 4 weeks. The presence or absence of viable spirochetes from tissues was confirmed by dark-field microscopy. Expectedly, blood samples from the mice infected with either host-adapted Δ VlsE Kan^r or Δ VlsE Gent^r were culture positive, whereas their bladder, heart, ear, and joint tissues were culture negative. All the murine tissues from the control mice infected with host-adapted WT Kan^r were culture positive for *B. burgdorferi* spirochetes. The results demonstrated that host-adapted mutant clones of *B. burgdorferi* were infectious at each superinfection.

Assessment of anti-*B. burgdorferi* immune response via a superinfection model. The experimental design included 90 C3H mice that were initially infected with the *B. burgdorferi* 297 strain. The infection was confirmed in all animals by culturing murine blood and ear tissues harvested at day 7 p.i. At day 14, 21, 28, 42, 56, or 70 p.i., actively infected mice (five animals per group) were subjected to secondary challenge (superinfection) by host-adapted WT Kan^r, Δ VlsE Kan^r, or Δ VlsE Gent^r. Finally, blood and other tissues (bladder, heart, ear, and joint) were harvested from each mouse at days 7 and 21 postsuperinfection, respectively, and then assessed for the presence of superinfecting spirochetes via culture. Individual tissues were placed in the respective antibiotic-containing BSK-II medium to select for the superinfecting clones. Each mouse had an ongoing *B. burgdorferi* 297-induced infection, as confirmed by positive culture of ear tissues harvested immediately prior to secondary challenge (data not shown).

At day 14 p.i., spirochetemia was established in at least 60% of the animals superinfected with either VlsE-competent (wild type) or -deficient (Δ VlsE) clones (Table 1). At day 21 p.i., 4 out of 10 mice exhibited spirochetemia induced by Δ VlsE Kan^r/ Δ VlsE

TABLE 2 Detection of superinfecting *Borrelia burgdorferi* in mouse tissues harvested at day 21 post-secondary challenge

Day of superinfection post-primary infection	Frequency of superinfecting clones detected in murine tissues ^a		
	WT Kan ^r	Δ VlsE Kan ^r	Δ VlsE Gent ^r
14	4/5	0/5	0/5
21	5/5	0/5	0/5
28	5/5	0/5	0/5
42	4/5	0/5	0/5
56	4/5	0/5	0/5
70	4/5	0/5	0/5

^aTissues include ear, heart, bladder, and joint. Values correspond to numbers of cultures positive/number of cultures tested.

Gent^r (Δ VlsE hereafter), whereas 0 out of 5 mice demonstrated WT Kan^r spirochetes in their blood. Overall, no statistical difference was observed between the WT Kan^r and Δ VlsE groups at either day 14 or 21 p.i. At day 28 p.i. both VlsE-deficient clones had the capacity to establish culture-detectable spirochetemia in 10 out of 10 mice. In contrast, WT Kan^r-induced spirochetemia was detected in only one mouse ($P = 0.0037$). As the primary infection further progressed, the number of mice with Δ VlsE-induced spirochetemia decreased from 50% (day 42 p.i.) to 0% (day 56 p.i.; $P = 0.0163$), indicating that the immune response became more protective against the VlsE-deficient clones, hence, non-VlsE surface antigens. The rate of WT Kan^r-induced spirochetemia remained relatively constant as superinfecting VlsE-competent spirochetes were detected in only 1 and 2 mice at days 42 and 56 p.i., respectively (Table 1). At day 70 p.i., however, 5 out of 5 mice were blood culture positive for WT Kan^r as opposed to only 2 out of 10 animals with Δ VlsE-induced spirochetemia ($P = 0.0070$). This observation demonstrates that the host antibody response against the VlsE-competent WT Kan^r clone was no longer protective at this late stage. This, in turn, may suggest that, in contrast to findings at day 28 p.i., VlsE was not the primary target of the immune response. Thus, outcomes of secondary challenge significantly varied between VlsE-competent and -deficient *B. burgdorferi* clones and were dependent on a stage of primary *B. burgdorferi* infection.

In order to examine whether superinfecting *B. burgdorferi* clones had the capacity to persist in superinfected mice and whether actively infected mice lacking culture-detectable spirochetemia had completely prevented superinfection, various murine tissues were harvested at day 21 post-secondary challenge (Table 2). As a result, no VlsE-deficient clones were cultured from any of the tissues, suggesting that spirochetemic mice ultimately prevented blood-borne dissemination of superinfecting VlsE-deficient *B. burgdorferi* clones. This is in contrast to WT Kan^r-challenged animals whose harvested tissues were positive for the superinfecting clone. This observation indicates that only the VlsE-competent *B. burgdorferi* had the capacity to establish long-term superinfection, which is consistent with the previous data (25). Detection of wild-type spirochetes in tissues of mice that lacked culture-detectable spirochetemia suggests that wild-type spirochetes were still present in the mouse blood upon secondary challenge, but at very low numbers.

Characterization of antibody response via serology. The data showed that the VlsE-competent and -deficient *B. burgdorferi* clones differ in their abilities to establish culture-detectable spirochetemia in mice actively infected with *B. burgdorferi*. This difference may be explained by variations in quantity and quality of the anti-*B. burgdorferi* antibody response over the course of *B. burgdorferi* infection. Therefore, in order to examine whether the antibody response was changing in strain 297-infected mice during the entire infection period, immune sera collected from animals at days 14, 21, 28, 42, 56, and 70 p.i. were analyzed by Western blotting (Fig. 1). The sera were blotted against whole-cell lysates of the 297, WT Kan^r, sVlsE, or Δ VlsE Kan^r clone and were reactive to a number of proteins, presumably including surface-localized antigens

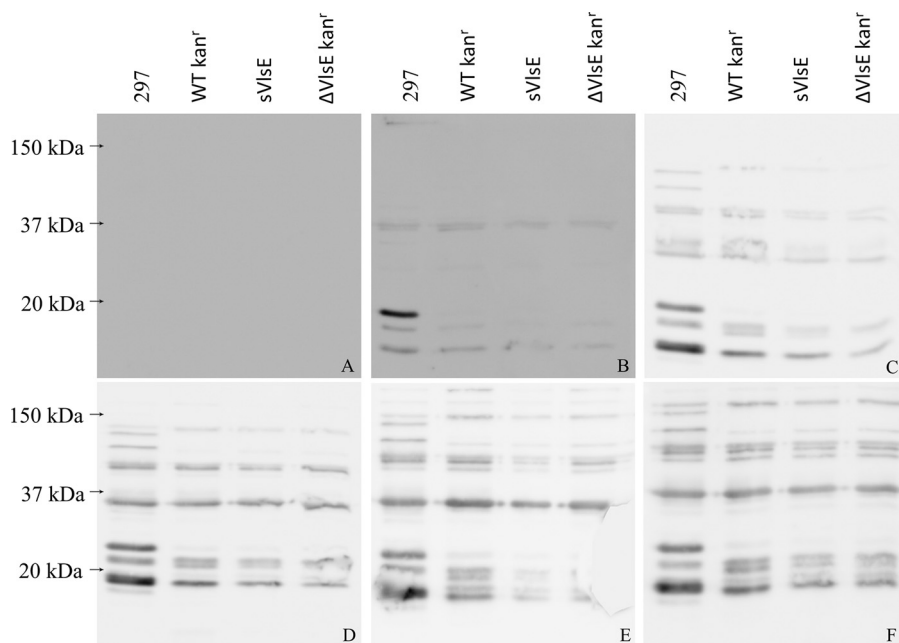


FIG 1 Analysis of anti-*B. burgdorferi* 297 immune sera by Western blotting. The whole-cell lysates of the 297, WT Kan^r, sVlsE, and ΔVlsE Kan^r (10⁶ cells per lane) clones were treated with preimmune sera collected from uninfected C3H mice (A) and anti-297 immune sera harvested from *B. burgdorferi*-infected C3H mice at days 14 (B), 28 (C), 42 (D), 56 (E), and 70 (F) postinfection. The blots can be compared to Coomassie blue-stained whole-cell lysates of the 297, WT Kan^r, sVlsE, and ΔVlsE Kan^r clones shown in Fig. S1 (17).

(32, 33). As expected, the lowest signal was observed in serum harvested at day 14 p.i., the time point at which anti-*B. burgdorferi* IgG antibody starts to appear in inbred mouse strains (34). The overall antibody response became more pronounced as the *B. burgdorferi* infection progressed in 297-infected mice. However, no noticeable difference could be noted for day 28 and 70 sera between the two clones (Fig. 1). In contrast to the three B31-derived clones, one prominent band was observed in the strain 297 lane at ~23 kDa. The 23-kDa band likely corresponds to outer surface protein C (OspC), which generates a strong antibody response (35). Given that there is 78% identity between strain 297 and B31 OspC (297-OspC and B31-OspC, respectively) amino acid sequences, it is possible that antibodies against 297-OspC do not react well with B31-OspC. Similarly, two additional bands in the 70- to 90-kDa range were prominent in the 297 lane but not in the other lanes (Fig. 1).

To further characterize the anti-297 antibody response, total serum amounts of different antibody isotypes, IgG1, IgG2a, and IgG2b, were quantified via enzyme-linked immunosorbent assays (ELISAs) (Fig. 2). During the first month of infection, IgG1 and IgG2b, but not IgG2a, were the dominant IgG isotypes. At a later stage of infection, day 70 p.i., the trend was reversed, indicating a shift from a Th2 to Th1 response. This finding is consistent with previous work, which demonstrated alike a Th1/Th2 imbalance in *B. burgdorferi*-infected C3H mice at week 5 p.i. (36).

Profiling of the antibody response via random peptide phage display libraries.

Random peptide phage display libraries (RPPDL) were utilized in the present study (37). Although RPPDL have been widely used for mapping epitopes (38–40), the tool was previously applied for epitope discovery of *B. burgdorferi* proteins by only two studies (41, 42). The necessity to sequence individual phage clones definitely limited RPPDL application in the past (43). The advent of next-generation sequencing (NGS), however, has allowed RPPDL to be widely applied for generating global profiles of antibody specificities (40).

To test whether during persistent *B. burgdorferi* infection antibody repertoires change over time, the present study involved both RPPDL and NGS. For that, the Ph.D.-7 phage library was utilized to detect any significant difference between antibody

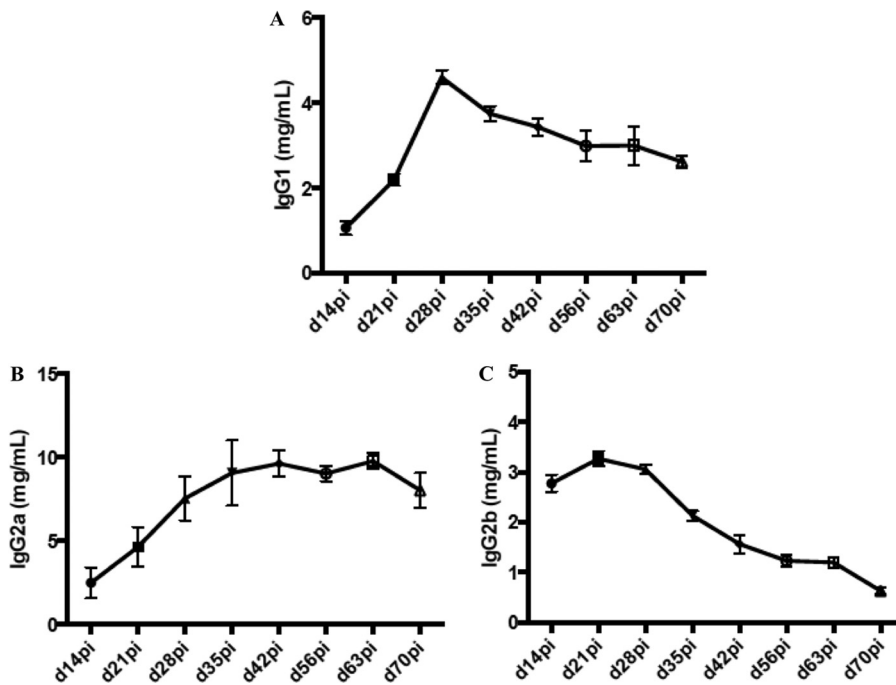


FIG 2 Quantification of total serum antibody isotypes in *B. burgdorferi* 297-infected C3H mice. Three C3H mice were subcutaneously challenged with *B. burgdorferi* 297 at 1×10^4 cells per mouse. Individual serum samples were collected weekly and then assessed, as indicated, for total IgG1, IgG2a, and IgG2b isotype-specific responses by ELISAs. d, day; pi, postinfection.

repertoires in immune sera collected at days 28 and 70 p.i. from individual 297-infected mice. As a result, approximately 1.4×10^5 distinct peptide sequences were generated from each anti-*B. burgdorferi* serum sample. The data analyses revealed that only 366 and 14 antibody specificities, respectively, were associated with the day 28 and day 70 serum samples. The difference between the two numbers was statistically significant, with a *P* value estimated via a permutation test being below 0.25%. However, when a multiple testing correction was applied, no significant difference in antibody repertoires was identified between the two time points of *B. burgdorferi* infection.

In order to compare anti-VlsE antibody responses between days 28 and 70 p.i., identified peptides were mapped to strain 297 VlsE (297-VlsE) or strain B31 VlsE (B31-VlsE) via blastp analysis (Fig. 3). The epitope mapping against the linear B31-VlsE structure showed no significant difference between the reactivities of day 28 and day 70 antibodies. Predicted cross-reactivity of anti-297-VlsE antibody to linear B31-VlsE is partially consistent with the anti-VlsE antibody reactivity of LD patients (44). Microarray-based epitope mapping demonstrated that IgG antibody of patients with chronic LD were mainly reactive to six peptides of B31-VlsE. The immunodominant epitopes were located within two invariable domains (VlsE residues 21 to 31, 61, 96, and 336 to 343) and one variable domain (VlsE residues 196 and 271 to 291). Consistently, reactivity of anti-297 antibody was predicted to invariant region 6 (IR6). IR6 has been shown to be highly immunogenic in humans, monkeys, and mice (22, 45, 46). In addition to IR6, C3H mice may also develop a strong antibody response to IR2 and IR4 (46). However, reactivity to these conserved regions was not pronounced in the present study (Fig. 3). Interestingly, IR2 and IR4 are not antigenic in humans and monkeys (46). The analysis also predicted strong reactivity of anti-297 antibody to the C-terminal invariable domain (amino acids 372 to 380) within the primary structure of 297-VlsE as opposed to that of B31-VlsE. Murine antibodies were consistently developed against the C-terminal region during the entire infection period as reactivity was detected in all mouse sera taken at day 28 and day 70 p.i. This fully supports the previous findings that the C-terminal invariable domain is highly immunodominant (20) but shows limited

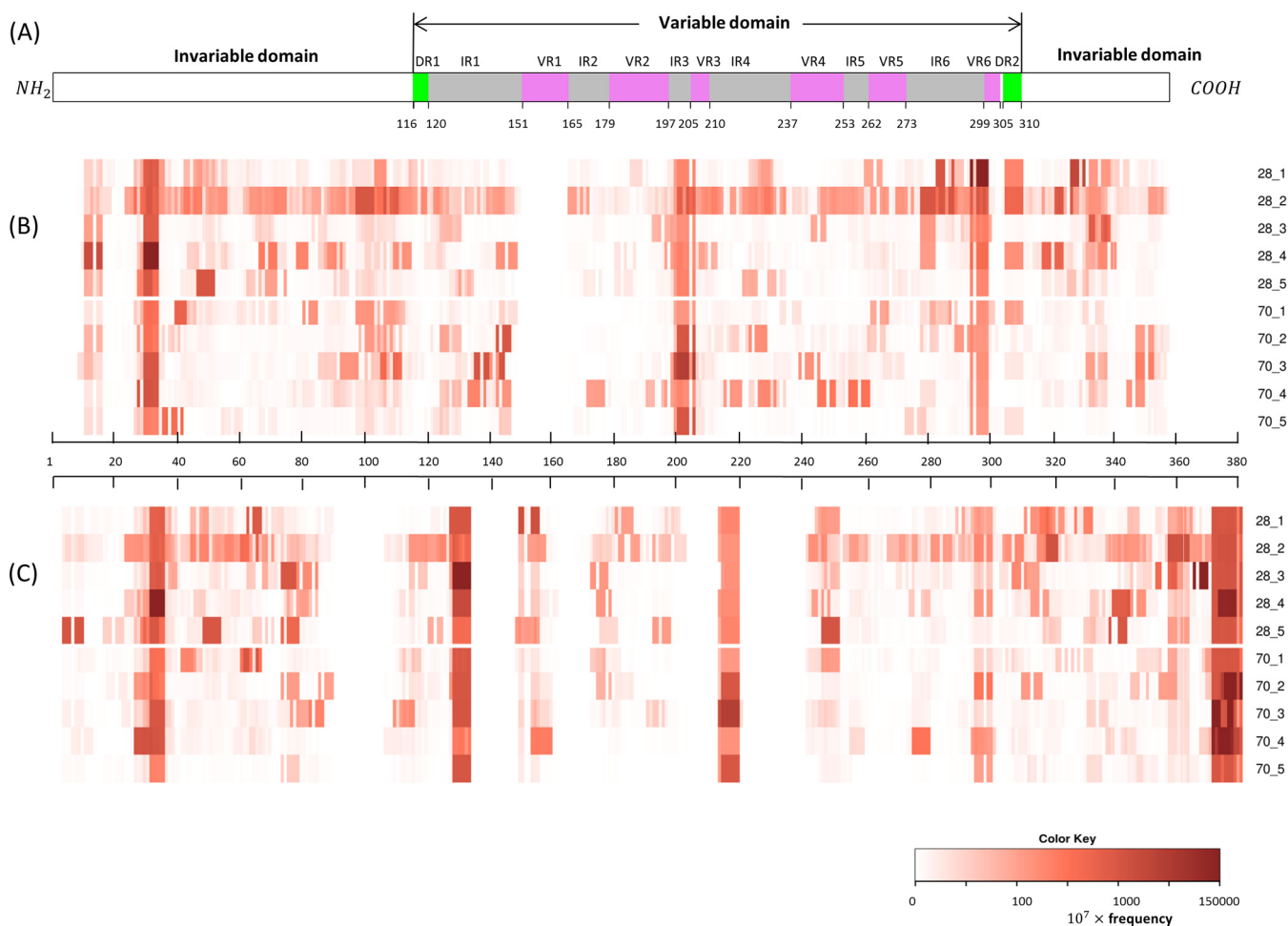


FIG 3 Epitope mapping of VlsE. Primary structure of B31-VlsE illustrating two direct repeats (DR1 and DR2; green) that demarcate one variable domain and two invariable domains. Shown are also six invariable (IR; gray) and variable (VR; pink) regions (59) (A). Heat maps were generated from predicted reactivity of anti-297 antibody to the primary structure of B31-VlsE (B) and 297-VlsE (C). Anti-297 sera were harvested from *B. burgdorferi* persistently infected C3H mice at days 28 and 70 postinfection (five animals per time point). The linear B31-VlsE structure is scaled to the B31-VlsE heat map.

antigenic conservation among *B. burgdorferi* strains (47). It was previously shown that the C-terminal domain sequences of 11 *B. burgdorferi* strains were not identical to the C-terminal sequence of the B31 strain (47). Likewise, due to a high degree of divergence with 46% identity and 53% similarity between B31-VlsE and 297-VlsE (8), strong antibody reactivity was predicted only to IR1 of 297-VlsE. Finally, no significant difference between the reactivities of day 28 and day 70 antibodies to the primary structures of translated cassettes *vls2* to *vls16* (*vls2-vls16*) (9) was identified (Fig. S2).

Similar to computational mapping of VlsE epitopes, identified peptides were also mapped to decorin-binding protein A (DbpA), decorin-binding protein (DbpB), and P35. These *B. burgdorferi* surface proteins were shown to be immunogenic and afforded protection in mice against *B. burgdorferi* infection (48–50). Consistently, no significant difference in antibody reactivities to contiguous epitopes of these immunogenic proteins was detected between day 28 and day 70 serum samples (Fig. S3).

DISCUSSION

A mathematical model previously developed as differential equations attempted to assess how duration of a bacterial infection may influence immune responses to variable and invariant surface antigens (24). It was calculated that antigenic variation of dominant surface antigen lengthens a bacterial infection to the point at which the immune response to invariant antigens becomes exhausted. In contrast, an immunodominant response to variable antigen does not predictably become exhausted but is,

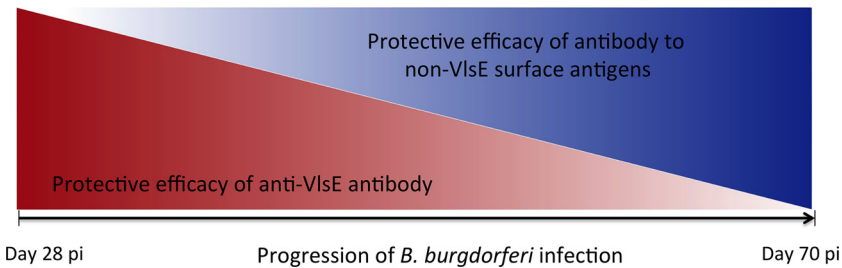


FIG 4 Dynamics of protective antibody responses directed against VlsE and non-VlsE surface antigens. The diagram shows how the protective efficacy of antibody responses changes in C3H mice infected with *B. burgdorferi* strain 297 over the course of *B. burgdorferi* infection. At the early stage of *B. burgdorferi* infection, day 28 postinfection (p.i.), the anti-*B. burgdorferi* antibody response to invariant (non-VlsE) surface epitopes (light blue) is not protective against the VlsE-deficient B31 A3 clone (Δ VlsE). The Δ VlsE clone is able to consistently establish culture-detectable spirochetemia in strain 297-infected C3H mice at this stage. In contrast, the anti-VlsE immune response (dark red) is sufficiently potent to block culture-detectable spirochetemia by a VlsE-competent B31 A3 clone. As the *B. burgdorferi* infection progresses, however, the immune response to non-VlsE surface epitopes becomes sufficiently strong (dark blue) to prevent spirochetemia by Δ VlsE (day 70 p.i.). Inversely, the anti-VlsE antibody response is no longer protective (light red) to clear culture-detectable superinfecting wild-type strain in the blood.

rather, transiently stimulated before the relevant antigenic variant is cleared (24). Therefore, it is possible that, during *B. burgdorferi* persistence, the antibody response to invariant (non-VlsE) surface epitopes is suppressed via direct or indirect involvement of highly variable and immunodominant VlsE proteins. If the model is correct, it is expected that the antibody response to invariant antigens will become nonprotective when *B. burgdorferi* infection is protracted in the host. To test the latter, changes in the protective efficacy of the immune response to non-VlsE surface antigens were monitored via the superinfection murine model.

In prior work, the superinfection *in vivo* assay demonstrated that the presence of the *vls* system may influence the ability of superinfecting *B. burgdorferi* clones to establish culture-detectable spirochetemia in persistently *B. burgdorferi*-infected mice (25). The superinfecting Δ VlsE clone established spirochetemia in three out of five 297-infected C3H mice, whereas wild-type spirochetes were not detected in any of five *B. burgdorferi*-infected animals (25). To confirm this observation, two independent experiments that involved isogenic VlsE-deficient clones, Δ VlsE Kan^r and Δ VlsE Gent^r, were reproduced in the present study. As a result, the data demonstrated that the Δ VlsE clones consistently exhibited the capacity to establish culture-detectable spirochetemia in 10 out of 10 animals at day 28 p.i., which statistically validates the earlier observation (25). The lack of culture-detectable spirochetemia by a VlsE-competent *B. burgdorferi* clone (wild type) consistently observed both in the previous (25) and current work suggests that, at this stage of *B. burgdorferi* infection, the protective antibody response was mainly targeting B31-VlsE variants and not the other surface antigens.

As the *B. burgdorferi* infection progressed, however, reversed outcomes were observed (Fig. 4). At day 70 p.i., the host immune response to non-VlsE antigens became sufficiently potent to clear spirochetemia by the Δ VlsE clone and yet failed to prevent WT-induced spirochetemia. At day 70 p.i., superinfecting wild-type *B. burgdorferi* established spirochetemia in all mice as opposed to a level of only 20% spirochetemia in animals infected with the Δ VlsE clone. This finding indicates that, over time, the infected mice were able to mount a more efficacious immune response against non-VlsE surface antigens. Moreover, at this later stage, the antibody response to B31-VlsE became inefficient, which may be accounted for by greater reactivity of a late antibody response to nonprotective VlsE epitopes via enhanced antigen processing (51). Furthermore, antibody reactivity to the membrane-proximal VlsE epitopes may sharply increase from early to late LD, yet these epitopes are inaccessible on intact spirochetes (51).

These observations taken together indicate that anti-*B. burgdorferi* antibody responses were quantitatively and qualitatively changing as the infection progressed

from day 28 through day 70 p.i. The antibody response became more pronounced and Th2 skewed in 297-infected C3H mice, as demonstrated by Western blotting and ELISA, respectively. These changes may partially account for the reversed outcomes of superinfection for both VlsE-competent and -deficient *B. burgdorferi* clones between the two time points of infection. It is also possible that, at this early stage, the antibody response to non-VlsE antigens was transiently weakened via temporal suppression of germinal center (GC) B cell responses (52). As early as 24 h p.i., *B. burgdorferi* spirochetes tend to colonize murine lymph tissues, which results in destruction of T and B cell zones (53, 54). The affected lymph nodes remain persistently infected with *B. burgdorferi* spirochetes (54, 55). Furthermore, germinal centers become structurally abnormal and fail to generate long-lived plasma and B cell memory cells for months (52). Thus, the present data indirectly support the idea that the LD pathogen requires only temporal suppression of the B cell response for fulfillment of its life cycle (52).

A possibility also exists that the observed dynamics in the protective efficacy of the anti-*B. burgdorferi* antibody response were due to changes in anti-*B. burgdorferi* antibody repertoires. For example, anti-*B. burgdorferi* antibody developed by day 28 p.i. could have been predominantly generated against VlsE epitopes, and, as the *B. burgdorferi* infection progressed, specificities of the anti-*B. burgdorferi* antibody were shifted toward non-VlsE surface antigens. To examine this possibility, global mimotope profiles derived from day 28 and 70 serum samples were compared to each other. Since mimotopes are peptides that may mimic both continuous and discontinuous antigens of a different nature (e.g., proteins, polysaccharides, and lipids) (56), the global mimotope comparison considered a variety of *B. burgdorferi* epitopes, including lipid and carbohydrate epitopes. However, the comparison revealed no major difference in antibody repertoires between the two time points of infection. Similarly, no significant changes were identified between the two antibody repertoires when identified mimotopes were mapped against primary structures of VlsE, DbpA, DbpB, and P35 (Fig. 3; see Fig. S3 in the supplemental material). Given, however, that many antibody epitopes of native proteins are discontinuous (57, 58), the mapping inherently underestimated repertoires of antibody developed to conformational epitopes of these *B. burgdorferi* surface proteins. Moreover, the BLAST-based analyses of antibody repertoires missed mimotopes that mimicked lipid and carbohydrate epitopes (43).

The present study also confirmed the previous observation that variable VlsE is a requirement for *B. burgdorferi* to establish persistent superinfection (25). Despite the variable ability of *B. burgdorferi* mutants to exhibit culture-detectable spirochetemia, only the *B. burgdorferi* clone with the intact *vls* system was consistently detected in various murine tissues harvested at day 21 postsuperinfection. In contrast, neither Δ VlsE clone introduced at different time points of *B. burgdorferi* infection was recovered from any of the tissues tested. Since *B. burgdorferi* is most likely required to invade the blood to reach various host tissues, the results suggest that a cross-protective anti-VlsE antibody response was not efficient at completely clearing the superinfecting spirochetes during the early stage of the primary infection. This consistent finding reiterates the importance of VlsE-mediated antigenic variation for persistent superinfection (25). Thus, the mathematical model, which predicted that antigenic variation of dominant surface antigen prolongs a bacterial infection to the point at which the immune response to invariant antigens is exhausted (24), may not apply to the LD pathogen. Therefore, based on the outcomes of day 70 superinfection, the tested hypothesis, which states that the protective efficacy of antibody response to *B. burgdorferi* non-VlsE surface antigens declines as *B. burgdorferi* infection progresses, can be rejected.

In summary, the study provides insights into a dynamic interplay between the host immune response and Lyme spirochetes in the context of VlsE antigenic variation. The data demonstrated that, during persistent *B. burgdorferi* infection, the host antibody response to *B. burgdorferi* invariant surface antigens could be transiently weakened and that, at this stage, variable VlsE might be the primary target of host immunity. Future studies aimed at identifying how and what *B. burgdorferi* antigens directly or indirectly

TABLE 3 *Borrelia burgdorferi* B31 clones used in the study

<i>B. burgdorferi</i> clone (description)	Genetic profile ^a		Reference or source
	<i>vls2-vls16</i>	<i>vlsE</i>	
B31 A3 lp25::kan (WT Kan ^r)	+	+	25
B31 A3 lp28-1Δ <i>vls</i> (Δ <i>VlsE</i> Kan ^r)	–	–	16
B31 A3 lp28-1Δ <i>vls</i> :: <i>vlsE</i> (s <i>VlsE</i>)	–	+	17
B31 A3 lp28-1Δ <i>vls</i> lp25:: <i>gent</i> (Δ <i>VlsE</i> Gent ^r)	+	+	This study

^aAll clones lacked plasmid cp9. *vls2-vls16* denotes silent cassettes of the *vls* locus.

manipulate host responses are warranted. Acquiring such knowledge may have direct implications in designing efficacious intervention strategies for LD patients.

MATERIALS AND METHODS

Ethics statement. The animal experimental procedures outlined in this work were performed under a Texas A&M University-approved animal use protocol. The animals were maintained at Texas A&M University in an animal facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Texas A&M University institutional policies and guidelines are in full compliance with the U.S. Public Health Service policy on humane care and use of laboratory animals.

Bacterial strains and culture conditions. B31 A3 lp25::kan (WT Kan^r) and B31 A3 lp28-1 Δ*vls*::*vlsE* (s*VlsE*) clones were generated and characterized in prior studies (25) and were generous gifts from Troy Bankhead (Table 3). The *B. burgdorferi* 297 strain and B31 A3 lp28-1 Δ*vls* (Δ*VlsE* Kan^r) clone, respectively, were kind gifts from Scott Samuels and George Chaconas by way of Troy Bankhead. All *B. burgdorferi* clones were cultivated in liquid Barbour-Stoenner-Kelly II (BSK-II) medium supplemented with 6% rabbit serum (Gemini Bio-Products, CA) and incubated at 35°C under 2.5% CO₂.

Mutant generation. To produce B31 A3 lp28-1 Δ*vls* lp25::*gent* (Δ*VlsE* Gent^r), the previously generated pAR15 plasmid was used to disrupt the *bbe02* gene localized on lp25 (NCBI reference sequence NC_001850.1) (25). This pJET1.2-derived plasmid contained the *bbe02* target (coordinates 361 to 4130) and the *flgBp*-driven gentamicin resistance gene (25). *B. burgdorferi* cells were electroporated according to a previously established protocol (16). In short, a total of 25 μg of DNA was used for electroporation. *Borrelia* cells were recovered at 35°C for 18 h and then diluted in 100 ml of prewarmed BSK-II medium supplemented with 100 μg ml⁻¹ gentamicin. The transformed cell suspension was aliquoted into 96-well plates and incubated at 35°C for 21 days. Genomic DNA was extracted from positive cultures utilizing a DNeasy blood and tissue kit (Qiagen, MD, USA). The insertion of a *flgBp-gent* cassette within the *bbe02* gene was confirmed by PCR using P168/169 primers (5'-CAGTTGCGCAGCCTGAATGG-3' and 5'-AGGTG GCGGTA CTGGGTGCG-3') as previously described (25). Finally, the plasmid profile of each PCR-positive clone was screened as described (11).

Murine infection. Male C3H/HeJ (C3H), C3SnSmn.CB17-Prkdc^{scid}/J (SCID), and BALB/cJ (BALB/c) mice of 4 to 6 weeks of age were obtained from Jackson Laboratories (ME, USA). The primary infection was performed on 4- to 6-week-old animals via subcutaneous inoculation of 1 × 10⁴ total spirochetes in the scapular region. The inoculum of each mutant clone of *B. burgdorferi* with a recombinant plasmid was first cultured in BSK-II medium containing an antibiotic, followed by dilution (approximately 1:1,000) with antibiotic-free BSK-II medium prior to murine infection. *B. burgdorferi* clones used for challenge were passaged *in vitro* no more than two times.

Secondary challenge (superinfection) was performed via subcutaneous transplantation of ear tissue (host-adapted *B. burgdorferi*) in the lumbar area as previously described (17, 31). In short, ear tissues were harvested from *B. burgdorferi*-infected SCID mice at day 21 postinfection (p.i.) and stored at -80°C until use. At the time of challenge ear pinnae were excised into small, circular pieces (2 mm in diameter) by a sterile ear punch and subcutaneously inserted via a skin incision in the lumbar region (two pieces per mouse). Given the identical sizes of ear tissues used for mouse challenges, the spirochetal loads of *B. burgdorferi* mutants were presumably similar. The infectivity of host-adapted *B. burgdorferi* was tested on naive C3H mice (three male mice per group). Specifically, the control mice were challenged with two ear pieces (2 mm in diameter) derived from two pinnae of each infected SCID mouse (one tissue piece from each ear) either at the time of or a few days after each superinfection (see Tables S2, S3, and S4 in the supplemental material).

Generation of host-adapted *B. burgdorferi* clones. Each mouse challenge was confirmed by culturing approximately 50 μl of blood aseptically drawn via maxillary bleed in 3 ml of BSK-II medium that contained a *Borrelia* antibiotic cocktail (0.02 mg ml⁻¹ phosphomycin, 0.05 mg ml⁻¹ rifampin, and 2.5 mg ml⁻¹ amphotericin B). Infection was monitored by culturing ear, heart, bladder, and tibiotarsal joint tissues aseptically harvested at days 7, 21, and 28 p.i. in BSK-II medium with the antibiotic cocktail. Blood or heart tissues were transferred into 8-ml polystyrene tubes (Becton Dickinson Labware, NJ, USA) containing 3 ml of BSK-II medium. Tissues of bladder, tibiotarsal joint, or ear were cultured in 1.7-ml polypropylene microcentrifuge tubes (Denville Scientific, Inc., MA, USA) with 1.0 ml of BSK-II medium. The tissues were incubated at 35°C under 2.5% CO₂ for up to 4 weeks. The presence or absence of viable spirochetes from tissues was confirmed by dark-field microscopy.

Western blot analysis. *B. burgdorferi* clones 297, WT Kan^r, s*VlsE*, and Δ*VlsE* Kan^r were grown in BSK-II medium to the late stationary phase. *B. burgdorferi* cells were counted, pelleted by centrifugation at

6,000 × *g* for 10 min at 4°C, and then washed twice with ice-cold phosphate-buffered saline (PBS). After PBS was removed, the cells were suspended in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer (100 mM Tris [pH 6.8], 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue) and incubated at 95°C for 10 min. Approximately 1 × 10⁶ cells were loaded onto a 15% acrylamide minigel (SDS-PAGE analysis is shown in Fig. S1). Then, resolved proteins were transferred onto polyvinylidene difluoride (PVDF) membrane with a pore size of 0.45 μm (Bio-Rad Laboratories, CA, USA). After the blot was blocked with 5% nonfat dry milk in PBS for 18 h at 4°C, it was then incubated in the same solution supplemented with mouse anti-297 immune or preimmune serum diluted 1:1,000 for 1 h. The immune serum samples were taken from 297-infected C3H mice at days 14, 28, 42, 56, and 70 p.i. At each time point blood was collected, and an equal amount of immune serum derived from five animals per time point was pooled and filter sterilized by passage through a 0.22-μm-pore-size syringe filter. Three naive C3H mice served as a source of preimmune sera. After four washes of 10 min each with PBS plus Tween 20 (PBST), the primary antibodies were detected using goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad Laboratories, CA, USA) diluted to 1:1,000 in Tris-buffered saline with Tween 20 (TBST) for 30 min. The blot was first washed three times in TBST for 10 min each and then once with nano-pure water. The blots were visualized using enhanced chemiluminescence (ECL) development.

ELISA. The immune sera were derived from 297-infected C3H mice at days 14, 28, 42, 56, and 70 p.i. as described above. Individual serum samples were tested by ELISAs to quantitate total IgG1, IgG2a, and IgG2b isotypes according to the manufacturer's instructions (eBioscience, Inc., CA, USA). Serum was diluted to 1:10,000 and plated at 4°C overnight. Samples were tested in duplicate, and each assay included preimmune serum as a negative control.

Generating serum antibody repertoire profiles using the Ph.D.-7 random peptide library. Twenty microliters of mouse serum and 10 μl of the Ph.D.-7 random peptide library (NEB, MA, USA) were diluted in 200 μl of Tris-buffered saline (TBST) buffer containing 0.1% Tween 20 and 1% bovine serum albumin (BSA) and then incubated overnight at room temperature. The phages bound to antibodies were isolated by applying 20 μl of protein G-agarose beads (Santa Cruz Biotechnology, Inc., TX, USA) to the phage-antibody mixture for 1 h. To eliminate unbound phages, the mixture with beads was transferred to a 96-well MultiScreen-Mesh filter plate (EMD Millipore, MA, USA) containing a 20-μm-pore-size nylon mesh on the bottom. Unbound phages were removed by applying a vacuum to the outside of the nylon mesh. The beads were washed four times with 100 μl of TBST buffer per well. Antibody-bound phages were eluted with 100 μl of 100 mM Tris-glycine buffer (pH 2.2). Then the buffer was replaced with 20 μl of 1 M Tris buffer (pH 9.1). The eluted phages were amplified by infecting bacteria according to the manufacturer's instructions. Amplified phages were subjected to two additional rounds of biopanning. Antibody-bound phages were isolated using protein G-agarose beads. DNA was isolated via phenol-chloroform extraction and ethanol precipitation. The 21-nucleotide (nt)-long DNA fragments coding random peptides were then PCR amplified using the following forward and reverse primers, respectively: 5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT (INDEX)TGGTACCTTTCTATTCTCACTCT-3' and 5'-CAAGCAGAAGAGGGCATAACGAGCTCTTCCGATCTAACAGTTTCGGCCGAACCTCCACC-3'. The INDEX in the sequence of the forward primer indicates a 6-nt barcode, which allows sequencing multiple libraries using a single line of the Illumina flow cell. For each mouse serum, a distinct forward primer with unique index sequence was used. The multiplexed PCR-amplified DNA library was then purified on agarose gel and sequenced using an Illumina HiSeq 2500 platform.

Next-generation data analysis. As a result of sequencing, a total of about 116 million DNA reads were obtained. Reads were demultiplexed based on the barcodes. Each read contained a unique index sequence of 6 nt in length and a 21-nt sequence coding a random peptide: 5'-(INDEX)GTGGTACCTTTC TATTCTCACTCT(21-nt sequence)G-3'. Then the 21-nt sequences were extracted from each read between positions 30 and 50 and translated to 7-mer peptides in the first frame (Text S1). Peptides that contained stop codons were not included in the analysis. The average number of all peptides per serum sample was approximately 1 × 10⁷. The number of distinct peptides identified was approximately 1.4 × 10⁵ per sample. The data were then analyzed via the Python programming language (Python Software Foundation [https://www.python.org]).

The strength of association between a peptide and a serum sample was measured as follows. A peptide, *P*, was associated with day 28 serum if *X*(*P*), the lowest frequency of *P* among day 28 serum samples, was higher than *Y*(*P*), the highest frequency of *P* among day 70 serum samples. The strength of association was then measured by the size of the gap: *X*(*P*) − *Y*(*P*). Similarly, a peptide was associated with day 70 serum samples if its smallest frequency among day 70 serum samples was higher than the highest frequency among day 28 serum samples.

Position coverage by peptides of VlsE and other *B. burgdorferi* proteins. For each serum sample, all peptides were mapped to VlsE of *B. burgdorferi* 297 (297-VlsE) (GenBank accession number AB041949.1) or B31 (B31-VlsE) (GenBank accession number AAC45733.1) strains using blastp with an identity threshold of 4 (i.e., only alignments with at least four exact amino acid matches were taken into account). For each VlsE position *X*, a peptide with the amino acid matched to position *X* and with *K* different VlsE matches contributed its frequency divided by *K* to the coverage of *X*. Overall, the coverage of *X*, *C*(*X*), was computed as the sum of contributions of all peptides matched to *X*. Similarly, all peptides were mapped to other *B. burgdorferi* surface proteins of the two *B. burgdorferi* strains, decorin-binding proteins A (DbpA), DbpB, and P35.

Statistical analysis. A one-tailed Fisher's exact test was used for comparison of mouse groups. A *P* value of <0.05 was considered significantly different. The statistical significance of the difference between the number of peptides associated with the day 28 serum and the number of peptides

associated with the day 70 serum was measured using a permutation test. The permutation test was used because of the comparatively small number of samples (five serum samples per time point). For each of the possible permutations, the difference between the numbers of associated peptides was found and compared with the actual difference.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00890-16>.

TEXT S1, PDF file, 0.1 MB.

TEXT S2, PDF file, 1.6 MB.

TEXT S3, PDF file, 15.7 MB.

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