The *Borrelia burgdorferi* VlsE Lipoprotein Prevents Antibody Binding to an Arthritis-Related Surface Antigen

**Highlights**

- Protection of a *B. burgdorferi* surface antigen from host antibodies is mediated by the antigenically variable VlsE lipoprotein

- Evidence supports a mechanism of epitope shielding by VlsE

- VlsE is not a universal protector against all anti-borrelial antibodies

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**In Brief**

Lone and Bankhead report that the antigenically variable VlsE protein of the Lyme disease agent *Borrelia burgdorferi* can prevent antibody binding to a surface antigen of the pathogen. They show that protection is likely via an epitope-shielding mechanism, thus expanding the current role of VlsE in immune evasion.

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The *Borrelia burgdorferi* VlsE Lipoprotein Prevents Antibody Binding to an Arthritis-Related Surface Antigen

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**SUMMARY**

Arp is an immunogenic protein of the Lyme disease spirochete *Borrelia burgdorferi* and contributes to joint inflammation during infection. Despite Arp eliciting a strong humoral response, antibodies fail to clear the infection. Given previous evidence of immune avoidance mediated by the antigenically variable lipoprotein of *B. burgdorferi*, VlsE, we use passive immunization assays to examine whether VlsE protects the pathogen from anti-Arp antibodies. The results show that spirochetes are only able to successfully infect passively immunized mice when VlsE is expressed. Subsequent immunofluorescence assays reveal that VlsE prevents binding of Arp-specific antibodies, thereby providing an explanation for the failure of Arp antisera to clear the infection. The results also show that the shielding effect of VlsE is not universal for all *B. burgdorferi* cell-surface antigens. The findings reported here represent a direct demonstration of VlsE-mediated protection of a specific *B. burgdorferi* surface antigen through a possible epitope-shielding mechanism.

**INTRODUCTION**

*Borrelia burgdorferi* is a gram-negative, motile flat-wave bacterium that causes Lyme borreliosis (Lyme disease), a tick-transmitted illness of humans and animals. The disease manifests in different clinical forms that range from a local rash termed erythema migrans (in humans) to systemic manifestations of fever and cardiac, neurological, and arthritic complications depending on whether the disease is in the early, disseminating, or late stage of progression (Bouchard et al., 2015; Hyde, 2017; Johnson et al., 2018; Murray and Shapiro, 2010; Stanek et al., 2012; Steere et al., 2016). Lyme disease is the most prevalent tick-borne disease in the United States, with nearly 30,000 new cases of the disease reported to the Centers for Disease Control and Prevention (CDC) annually (CDC, 2019; Hinckley et al., 2014). However, the incidence has been suggested to be up to 10-fold higher due to misdiagnosis and failure to report the disease (Kuehn, 2013; Wright et al., 2012).

Among the different clinical manifestations of late-stage Lyme disease in the United States, arthritis is the most common, with nearly 60% of late-stage Lyme disease patients reporting arthritic complications (CDC, 2019). The arthritis-related protein (Arp) of the *B. burgdorferi* B31 strain is a surface lipoprotein encoded by the *bbf01* locus of the 28-kb linear plasmid, Ip28-1, and has been shown to be upregulated during host infection (Feng et al., 2000; Liang et al., 2004). Previous studies have demonstrated that Arp is associated with the development of arthritis and contributes to joint swelling in mice (Hove et al., 2014; Imai et al., 2013), and anti-Arp antibodies have been shown to resolve *B. burgdorferi*-induced arthritis in severe combined immunodeficiency (SCID) mice (Barthold et al., 2006; Feng et al., 2000; Feng et al., 2003). However, Arp-specific antibodies have been shown to be unable to prevent or clear *B. burgdorferi* infection in mice (Barthold et al., 2006; Feng et al., 2003). This inability of anti-Arp antibodies to clear *B. burgdorferi* infection could be due to epitope shielding by another surface protein capable of immune evasion. In *B. burgdorferi*, VlsE is an antigenically variable cell-surface protein that evades the antibody response by continuously changing its surface epitopes (Norris, 2000), and it could potentially function to shield Arp from antibodies.

The VlsE protein undergoes antigenic variation exclusively during mammalian infection (Norris, 2006; Zhang et al., 1997; Zhang and Norris, 1998) and has been reproducibly shown to be required for *B. burgdorferi* survival and persistence while in the presence of a host humoral response (Bankhead, 2016; Bankhead and Chaconas, 2007; Lawrenz et al., 2004; Rogovsky and Bankhead, 2013; Rogovsky and Bankhead, 2015). The antigenic variability of VlsE results from nonreciprocal recombination between the *vls*E gene and 15 silent *vls* cassettes within the *vls* locus located on the same Ip28-1 plasmid that carries *arp* (Norris, 2006; Palmer et al., 2009; Verhey et al., 2018a, 2018b; Zhang et al., 1997). Similar to Arp, VlsE has been shown to be upregulated during host infection (Liang et al., 2004). A long-standing question has been how *B. burgdorferi* immune escape is accomplished through sequence variation of this single lipoprotein, despite the presence of a substantial number of additional antigens residing on the bacterial surface. A role for VlsE besides antigenic variation is not currently known, but it has been proposed that the protein might function in other forms of immune evasion (Liang et al., 2002; Philipp et al., 2001). Among the several models that have been suggested, one scenario proposes that VlsE may act as a shield to obscure the epitopes of other surface antigens (Bankhead, 2016).
Given the above information, we hypothesized that VlsE may function to protect Arp from an antibody-mediated immune response. We report here that passive immunization assays involving SCID mice showed that anti-Arp antibodies prevent *B. burgdorferi* infection only in clones that lack expression of VlsE, suggesting immune protection by the antigenically variable lipoprotein. We also performed immunofluorescence experiments to determine whether anti-Arp antibodies bind to Arp-expressing *B. burgdorferi* strains in the presence of VlsE. These experiments confirmed that Arp is indeed protected from Arp-specific antibody binding when VlsE is present, likely via an epitope-shielding mechanism.

**RESULTS**

**Generation of a *B. burgdorferi* Clone Capable of Simultaneous Expression of Arp and VlsE**

Previous work has provided some evidence of VlsE-mediated protection of *B. burgdorferi* surface antigens against the host antibody immune response (Rogovskyy and Bankhead, 2013, 2014; Rogovskyy et al., 2015), although any surface proteins being directly protected by VlsE had yet to be identified. Despite the inability of Arp antiserum to prevent or clear infection by *B. burgdorferi* in mice (Barthold et al., 2006; Feng et al., 2003), a pilot experiment in our lab demonstrated that a vlsE-deletion mutant clone of *B. burgdorferi* could be successfully cleared in infected SCID mice after treatment with anti-Arp antibodies (unpublished data). This preliminary finding was the basis for initiating studies to determine whether the presence of VlsE allows for evasion of the Arp surface antigen from recognition by anti-Arp antibodies.

Our initial strategy to test this was to treat SCID mice with sera containing anti-Arp antibodies and then challenge mice with *B. burgdorferi* clones expressing or lacking VlsE. For this approach to be successful, it was necessary that our *B. burgdorferi* clone exhibit simultaneous expression of Arp and VlsE prior to murine challenge. Although western blot analysis showed some VlsE surface expression from in-vitro-grown wild-type B31-A3 spirochetes similar to a previously published study (Figure 1A; Grimm et al., 2004), no detectable Arp could be observed from this clone (Figure 1B). To achieve either individual or simultaneous expression of Arp and VlsE, we employed a strategy that involved the generation of high-copy expression clones of the *B. burgdorferi* B31-A1 strain that lacks the *arp* and vlsE-resident Ip28-1 plasmid (hereafter referred to as A1arp+/vlsE−; Elias et al., 2002). This was accomplished by cloning *arp* or vlsE into a pBSV2 shuttle vector (Stewart et al., 2001) harboring either a gentamicin (pBSV2g-arp) or kanamycin (pBSV2-vlsE) resistance gene, respectively, and then transforming the plasmids into A1arp+/vlsE− cells. Because these genes reside on a high-copy shuttle vector with only their basal promoter sequences intact, the expectation was elevated expression levels of *arp* and vlsE that are no longer subjected to regulatory effects.

To ensure that the generated A1 clones (Tables S1 and S2) express and membrane localize Arp (A1arp+/vlsE−), VlsE (A1arp+/vlsE−), or both Arp and VlsE (A1arp+/vlsE−), western blot analysis was conducted on proteinase-K-treated spirochetes and Triton X-114 extracted spirochete proteins. As expected, immunoblots using anti-Arp or anti-VlsE antibodies confirmed both individual and simultaneous surface expression of the two lipoproteins compared to the parental A1arp+/vlsE− control (Figures 1C–1F). Western blot analysis utilizing antibodies against the cytosolic BosR protein verified proper phase separation of detergent and aqueous phase proteins (Figure 1G).

Because Arp and VlsE in the A1arp+/vlsE− strain are expressed from plasmids with different antibiotic resistance markers but identical origins of replication, it was also necessary to determine whether individual bacteria produce the Arp protein to be targeted on their surface. To ascertain Arp expression on individual spirochetes harboring pBSV2g-arp, flow cytometric analysis was carried out on the A1arp+/vlsE− clone and a control strain that harbored the pBSV2g-arp plasmid along with the same pBSV2 plasmid found in the A1arp+/vlsE− clone, except that it lacked the vlsE gene. The absence of the vlsE gene was necessary due to the potential of VlsE to prevent binding of anti-Arp antibodies during flow cytometric analysis. The results show that although the presence of both plasmids leads to a ~2-fold decrease in the median fluorescence intensity (MFI; 365 versus 172; Figures 1H and S1), Arp is clearly expressed within the population at levels that would allow targeting by anti-Arp antibodies. In comparison to Arp expression, VlsE expression was higher in both A1arp+/vlsE− and A1arp+/vlsE+ (Figure S2); similar to Arp however, VlsE expression was found to be lower in the A1arp+/vlsE+ clone (MFI681 versus 500). Combined, the above results demonstrate that the combination of both constructs in the A1arp+/vlsE+ clone leads to an increase in the overall expression of Arp or VlsE when compared to the B31-A3 wild-type strain.

Expression of Arp and/or VlsE by the various clones during infection of C3H mice was confirmed by the presence (Figures 1I–1K) or absence (Figure 1L) of serum antibodies against these two proteins. Interestingly, the antibody response to multiple proteins appeared to differ somewhat between sera raised in the presence or absence of VlsE (Figure 1J versus Figure 1K), potentially indicating that VlsE might shield multiple surface antigens. For the remainder of this study, the A1arp+/vlsE−, A1arp+/vlsE+, and A1arp+/vlsE+ clones were utilized to assess the ability of VlsE to provide protection of Arp from antibody recognition. It should also be noted that VlsE antigenic variation cannot occur in these clones due to the lack of the silent cassette region. However, the prediction was that vlsE recombination would not be necessary to assess VlsE-mediated immune protection of surface antigens due to the use of passively transferred antibodies.

**Arp-Specific Antiserum Prevents Infection by *B. burgdorferi* Expressing Arp, but Not by *B. burgdorferi* Expressing Both Arp and VlsE**

To assay whether VlsE has the capacity to protect Arp from anti-Arp antibodies, SCID mice were divided into nine groups. Groups of mice were passively immunized with either sera raised against recombinant Arp, the A1arp+/vlsE− clone, or non-immune sera and then challenged 16 h later with A1arp+/vlsE−, A1arp+/vlsE+, or A1arp+/vlsE+ spirochetes. Blood samples were collected at 7 days post-inoculation, and ear, heart, bladder, and joint tissue samples were harvested at 28 days post-inoculation. All samples
In Vitro and In Vivo Expression of Both Arp and VlsE and Their Surface Localization by B. burgdorferi Clones

(A and B) Western blots demonstrating in vitro VlsE and Arp expression by the wild-type B31-A3 strain and B31-A1 derived clones transformed with circular plasmid vectors carrying vlsE and arp. Recombinant Arp (rArp) and VlsE (rVlsE-MBP) served as positive controls. The blots were probed with either (A) anti-VlsE (aVlsE) or (B) anti-Arp antibodies (aArp).

(C and D) Cell-surface localization of VlsE and Arp in clones treated with or without proteinase K (pk). Western blot of (C) A1arp+/vlsE+ and A1arp+/vlsE- cell lysates probed with aVlsE antibodies and (D) A1arp+/vlsE+ and A1arp+/vlsE- cell lysates probed with aArp antibodies are shown.

(E–G) Membrane localization of VlsE and Arp. The membrane lipoproteins (M) were separated in Triton X-114 detergent phase and non-membrane proteins (N) in aqueous phase.

(H) Flow cytometric analysis of Arp expression in A1arp+/vlsE- strain, which harbors an arp-expressing plasmid (pBSV2-arp), and a control strain (A1arp+/vlsE- pBSV2-ponC) that contains an additional plasmid, pBSV2-ponC.

(I and J) In vivo expression of VlsE and Arp. In vivo expression of (I) VlsE and (J) Arp was confirmed via western blot analysis of A1arp+/vlsE+ and A1arp+/vlsE- cell lysates probed with sera raised against A1arp+/vlsE+ spirochetes.

(K) In vivo expression of Arp by A1arp+/vlsE+ spirochetes was confirmed by probing A1arp+/vlsE+ cell lysate with sera raised against the A1arp+/vlsE+ clone. (Higher amounts of rArp have been loaded in the control lane, hence the brighter band.)

(L) Western blot of cell lysates of A1arp+/vlsE+, A1arp+/vlsE-, and A1arp+/vlsE- probed with A1arp+/vlsE- antisera served as a control for specificity of Arp- and VlsE-specific antibodies in the antisera raise against A1arp+/vlsE+ and A1arp+/vlsE- clones. Numbers on the left correspond to approximate molecular weight in kilodaltons. Sera was pooled from multiple mice, and similar dilutions of sera were used in each panel.
were cultured individually under appropriate antibiotic selection, and the presence of the arp and/or vlsE-harboring shuttle plasmids in recovered spirochetes was verified via PCR analysis.

As expected, the results showed that A1arp+/vlsE− mids in recovered spirochetes was verified via PCR analysis. However, Arp antisera were able to prevent infection which exact group differs from another. Arp antisera were able to prevent infection by A1arp+/vlsE− spirochetes lacking Arp (Tables 1 and S3). However, Arp antisera were unable to prevent infection by A1arp+/vlsE− spirochetes lacking Arp (Tables 1 and S3). Because the difference between the A1arp+/vlsE− and A1arp+/vlsE+ clones is the production of VlsE, this finding strongly suggests that the presence of VlsE protects the Arp lipoprotein from host antibody recognition. These results also demonstrate successful prevention of challenge of Arp-expressing spirochetes by Arp antisera, suggesting that past failures were at least partially due to a lack of Arp expression by cultivated wild-type clones prior to challenge of mice. Interestingly, the A1arp+/vlsE+ clone was mostly incapable of infecting SCID mice immunized with anti-A1arp−/vlsE− antibodies (Table 1). This latter result suggests that the anti-A1arp−/vlsE− serum contains antibodies against B. burgdorferi surface antigens that are not protected by VlsE.

The in-vitro-grown B31-strain-derived spirochetes utilized in the above passive immunization assays might display some surface antigens that would not normally be expressed during host infection and thus may not be typically protected by the presence of VlsE. This could potentially account for the lack of universal protection exhibited by the VlsE. Additionally, the possibility exists that other host-specific surface proteins of B. burgdorferi may also be able to protect Arp from host antibodies. For these reasons, we decided to challenge passively immunized mice with “host-adapted” spirochetes via an infected ear tissue transplantation methodology (Rogovskyy and Bankhead, 2013; de Silva et al., 1998). To do this, B.-burgdorferi-infected ear biopsy tissue was transplanted into immunized SCID mice. This approach allowed us to test the protective efficacy of Arp antisera in a more physiologically relevant model of infection. The results showed that Arp antisera were able to prevent infection by A1arp+/vlsE+ spirochetes, whereas Arp antisera were unable to prevent infection by A1arp+/vlsE− Arp+/vlsE+ clones after passive immunization with sera non-immune sera 7/9 6/6 6/6 0.4857

Table 1. Arp-Specific Antiserum Prevents Infection by B. burgdorferi Expressing Arp, but Not by B. burgdorferi Expressing Both Arp and VlsE

| Antiserum Treatment | A1arp+/vlsE+ | A1arp+/vlsE− | A1arp−/vlsE− | p Valuea |
|---------------------|-------------|-------------|-------------|---------|
| Arp antisera        | 2/9         | 9/9         | 8/9         | 0.0009  |
| A1arp−/vlsE+ antisera | 0/6       | 1/6         | 0/6         | 1.0000  |
| Non-immune sera     | 7/9         | 6/6         | 6/6         | 0.4857  |

*aThe table shows the proportion of mice (infected/total) successfully infected by B. burgdorferi clones after passive immunization with sera shown in the first column. An animal was considered successfully infected if spirochetes were detected from any of the tissues, including ears, heart, bladder, or joints, at 28 days post-challenge. Significance was calculated using Fisher’s exact test (last column) to examine whether a statistically significant difference exists across all groups (proportions), while Bonferroni correction was applied to test which exact group differs from another. Significant at p < 0.05 after applying Bonferroni correction for multiple comparisons between groups.
via subcutaneous stab incision into passively immunized SCID mice. Similar to the previous experiments utilizing in-vitro-grown spirochetes, the results showed that anti-Arp antibodies were able to prevent infection by the host-adapted $A1_{arp^+/vlsE^-/C0}$ clone despite the presence of upregulated mammalian host-associated surface proteins in these spirochetes (Table S4). In contrast, the host-adapted $A1_{arp^-/vlsE^-/C0}$ parent clone was able to infect sera-treated mice, thereby confirming the binding of anti-Arp antibodies to Arp epitopes present on the surface of these spirochetes (Figure 2A). Importantly, the anti-Arp antibodies were specific to the Arp antigen, as these antibodies were unable to detect the $A1_{arp^-/vlsE^-}$ parent clone lacking both Arp and VlsE. As predicted, $A1_{arp^-/vlsE^-}$ spirochetes were undetectable by immunofluorescence using these same antisera (Figure 2A), indicating that VlsE indeed shields Arp epitopes from anti-Arp antibody recognition and binding. To confirm that only surface-expressed proteins were targeted by the anti-Arp antibodies, $A1_{arp^-/vlsE^-}$ was probed with antibodies raised against the periplasmic flagellar protein FlaB. Indeed, anti-FlaB antibodies could only access the periplasmic flagellar protein when spirochetes were first permeabilized with methanol (Figure 2B). Finally, all $B. burgdorferi$ strains could be detected when $A1_{arp^-/vlsE^-}$ antisera were used, implying that not all surface antigens are shielded by VlsE (Figure 3). These results corroborate the findings obtained from the mouse challenge assays (Tables 1 and S4), including those suggesting that VlsE-mediated protection does not encompass all proteins expressed on the $B. burgdorferi$ cell surface.

**DISCUSSION**

Numerous studies over the years have demonstrated the importance of VlsE antigenic variation for immune evasion and persistence during host infection by the Lyme disease pathogen. However, the question remained how antigenic variation of a single protein could confer protection to $B. burgdorferi$ from antibodies that are known to be generated against a large number of proteins expressed on the spirochete cell surface. The studies presented here are a continuation of work by our lab demonstrating the presence of a VlsE-mediated immune avoidance system that allows for evasion of non-VlsE surface antigens from the host antibody response (Bankhead, 2016; Bankhead and Chaconas, 2007; Rogovskyy and Bankhead, 2013; Rogovskyy et al., 2015).

In the current study, we demonstrate that Arp antisera can prevent infection of mice by $B. burgdorferi$ expressing Arp and...
lacking VlsE, thus corroborating earlier studies suggesting that Arp is an immunodominant protein (Hodzic et al., 2002; Tunev et al., 2011). However, these same antibodies were unable to prevent infection by either in-vitro-grown or host-adapted spirochetes expressing both Arp and VlsE, indicating that VlsE protects Arp against host antibodies. Moreover, the results demonstrating that the host-adapted A1arp+/vlsE+ clone was able to successfully infect anti-Arp-sera-treated mice indicate that the protective effects may be specific to VlsE. However, in the absence of another surface-protein-expressing plasmid isogenic to the VlsE-producing plasmid presented in this study, the possibility cannot be ruled out that overexpression of other lipoproteins might also protect Arp. Additional studies will be needed in order to determine whether immune protection of Arp is truly a specific function mediated by VlsE.

The likelihood that the observed immune protection by VlsE is via epitope shielding is demonstrated by the inability of Arp-specific antibodies to bind spirochetes that express VlsE in immunofluorescent assays. One caveat that deserves mention is that the in vitro expression levels of both Arp and VlsE are normally high in the engineered clones, and whether this has any bearing on how VlsE and Arp interact during infection by wild-type spirochetes is difficult to assess. However, it can be argued that increased expression of Arp would normally be expected to circumvent any potential shielding effects by VlsE. For this reason, we feel that our results strongly argue in favor of VlsE providing protection to Arp given the high expression levels of this protein. An additional caveat is that the A1arp+/vlsE+ spirochetes lack the lp25 plasmid, which is still present in the A1arp+/vlsE+ clone. This plasmid was lost after transformation of cells with pBSV2-vlsE but still retains infectivity due to the presence of the pncA gene harbored on this same plasmid. Although any effects on the prevention of binding by anti-Arp antibodies cannot be ruled out, we believe this genetic difference is irrelevant due to the specificity in binding of the Arp antisera to the A1arp+/vlsE+ spirochetes in comparison to the parental A1arp+/vlsE+ clone, both of which contain lp25.

Arp is a highly conserved protein (95%–100% identity) among B. burgdorferi sensu stricto strains (Imai et al., 2013), which are primarily responsible for cases of Lyme disease in the United States (Heymann and Ellis, 2012). Apart from its role in Lyme disease arthritis (Feng et al., 2003), the biological function of Arp during host infection by B. burgdorferi is not currently known. Nevertheless, being a highly conserved protein and an immunodominant antigen, Arp would likely render B. burgdorferi amenable to complete elimination if it were not protected by the immune-evasive and antigenically variable VlsE protein. This would also offer a more cost-effective means of immune evasion: devote one protein to evade an adaptive immune response against other important proteins that need to be conserved for better survival of the pathogen.

In summary, we now demonstrate that Arp is shielded by VlsE from host antibodies, providing an explanation as to why anti-Arp antibodies were previously found to be unable to clear spirochetes during murine infection (Barthold et al., 2006; Feng et al., 2003). The findings presented here identify one of potentially multiple proteins that are protected through VlsE-mediated immune evasion and provide direct evidence of epitope shielding by the VlsE protein. The ability to assay for protection of Arp will now allow for experimental assays aimed at dissecting the mechanistic aspects of VlsE-mediated immune protection. It is also clear that VlsE is not a universal protector against all the anti-borrelial antibodies. Future studies will be necessary to determine how VlsE can sustain B. burgdorferi persistence during host infection in spite of the fact that it does not provide protection against all borrelial surface antigens.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.02.081.

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**AUTHOR CONTRIBUTIONS**

Conceptualization, A.G.L. and T.B.; Methodology, A.G.L. and T.B.; Investigation, A.G.L.; Validation, A.G.L.; Formal Analysis, A.G.L. and T.B.; Writing – Original Draft, A.G.L. and T.B.; Writing – Review & Editing, A.G.L. and T.B.; Visualization, A.G.L. and T.B.; Funding Acquisition, T.B.; Resources, T.B.; Supervision, T.B.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.
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### STAR METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies** | | |
| Alexa Fluor 647 Anti-mouse IgG | Thermo Fisher Scientific | Cat# A-31571; RRID:AB_162542 |
| Alexa Fluor 647 Anti-Rabbit IgG | Abcam | Cat# ab150075; RRID:AB_2752244 |
| Anti-A1arp/vlsE⁻ | This paper | N/A |
| Anti-A1arp⁺/vlsE⁻ | This paper | N/A |
| Anti-A1arp⁺/vlsE⁺ | This paper | N/A |
| Anti-Arp antibody | This paper | N/A |
| Anti-BosR IgG | General Biosciences | Cat# AB76001-200; Clone 2F9-D2. |
| Anti-FlaB antibody | Rockland | Cat# 200-401-C14; RRID:AB_10703395 |
| Anti-mouse IgG | Abcam | Cat# ab6789; RRID:AB_955439 |
| Anti-rabbit IgG | Abcam | Cat# ab6802; RRID:AB_955445 |
| Anti-VlsE IgG | Rockland | Cat# 200-401-C33; RRID:AB_10924424 |
| **Bacterial and Virus Strains** | | |
| B. burgdorferi A1arp⁻/vlsE⁺ | This paper | N/A |
| B. burgdorferi A1arp⁺/vlsE⁻ | This paper | N/A |
| B. burgdorferi A1arp⁺/vlsE⁺ | This paper | N/A |
| B. burgdorferi B31-A1 (A1arp⁺/vlsE⁺) | Elias et al., 2002 | N/A |
| B. burgdorferi B31-A3 | Elias et al., 2002 | N/A |
| E. coli Rosetta (DE3) pLysS | Novagen | Cat# 70956 |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| Amphotericin B | Sigma | Cat# A9528; CAS: 1397-89-3 |
| Betadine Surgical Scrub | Purdue Products | Cat# 516163 |
| Blocking buffer | Abcam | Cat# ab126587 |
| Clarity Western ECL | Bio-Rad Laboratories | Cat# 170-5060 |
| Gentamycin | Sigma | Cat# G1264; CAS: 1405-41-0 |
| Isopropyl β-D-1-thiogalactopyranoside | Novagen | Cat# 70527-3; CAS: 367-93-1 |
| Kanamycin | Sigma | Cat# K1377; CAS: 25389-94-0 |
| Methanol | Sigma | Cat# 34860 |
| Oriole Fluorescent Gel Stain | Bio-Rad Laboratories | Cat# 1610496; |
| Phosphomycin | Sigma | Cat# P5369; |
| Prolong Gold Antifade | Thermofisher Scientific | Cat# P36941 |
| Rifampicin | Sigma | Cat# R3501; CAS: 13292-46-1 |
| Syto 9 | Thermofisher Scientific | Cat# S34854 |
| Triton X-114 | Sigma | Cat# X-114; CAS: 9036-19-5 |
| Tyrode’s Solution | Boston Bioproducts | Cat# PY-921 |
| VlsE Control Protein | Rockland | Cat# 000-001-C33 |
| **Critical Commercial Assays** | | |
| Coomassie Plus Assay Kit | Thermofisher Scientific | Cat# 23236 |
| Melon Gel IgG Purification Kit | Thermofisher Scientific | Cat# P145206 |
| Ni-NTA Purification System | Thermofisher Scientific | Cat# K95001 |
| Wizard Genomic DNA Purification Kit | Promega | Cat# A1120, |

(Continued on next page)
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Troy Bankhead (tbankhead@wsu.edu). All unique/stable reagents generated in this study are available from the Lead Contact without restriction.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

B. burgdorferi strains and culture conditions

Clones described in this study (Table S1) were generated from the B31-A1 strain (Elias et al., 2002). The plasmid profiles of these clones are shown in Figure S3, and comparison among the plasmid profiles in Table S2. All B. burgdorferi strains were grown at 35°C under 1.5% CO2 tension in Barbour-Stoenner-Kelly II (BSK-II) medium supplemented with 6% rabbit serum and appropriate antibiotics as required (gentamicin 100 µg/ml and/or kanamycin 100 µg/ml). Cell densities and growth phase were monitored by dark-field microscopy and enumerated using a Petroff-Hausser counting chamber.

Mice

For in vivo studies, four to five-week-old male C3H SCID (C3SnSmn.CB17-Prkdcscid/J, Jackson Laboratory) were subcutaneously inoculated with 100 µl of sera raised either against the A1arp/vlsE clone or against recombinant Arp, and then infection with the appropriate clone of B. burgdorferi was followed 16 hours later. Clones were passaged no more than two times in vitro from frozen glycerol stocks prior to murine challenge. Tissue were collected for spirochete presence or absence as described in Method Details.

For infection of mice via tissue transplantation, 3 mm diameter discs of ear tissue containing host-adapted borrelia were transplanted into the lumbar region of SCID mice. Ear (pinnae) tissues were harvested from SCID mice at 21 days post infection. Mice were anesthetized with isoflurane, and their ears were shaven, disinfected with Betadine Surgical Scrub (Purdue Products, Stamford, CT), and then briefly washed with isopropyl alcohol followed by rinsing with sterile water and dried with sterile gauze. Mice were euthanized to harvest ears, which were cut into 3 mm diameter discs using a biopsy punch tool. The discs were immediately
transplanted underneath the skin via a small stab incision in a shaven surgical clean lumbar region. To verify that the tissue explants contained live spirochetes, a piece of 3 mm ear tissue from each mouse was incubated in BSK-II medium supplemented with a mixture of antibiotic and antifungal drugs as described above. The presence or absence of spirochetes in mice that were transplanted with infected tissue was determined by culture of tissues and microscopy as described below.

**Ethics statement**

The experiments on mice were carried out according to the protocols and guidelines approved by American Association for Accreditation of Laboratory Animal Care (AAALAC) and by the Office of Campus Veterinarian at Washington State University (Animal Welfare Assurance A3485-01 and USDA registration number 91-R-002). The animals were housed and maintained in an AAALAC-accredited facility at Washington State University, Pullman, WA. The Washington State University Institutional Animal Care and Use Committee approved the experimental procedures carried out during the current studies.

**METHOD DETAILS**

**Generation of Arp- and VlsE-expressing clones**

The *B. burgdorferi* A1arp+/vlsE- clone was generated by transforming B31-A1 cells with a pBSV2g (Elias et al., 2003) shuttle plasmid containing *arp* gene (corresponding to coordinates 68 to 1605 of lp28-1) and its native promoter (pBSV2g-arp). The plasmid also carries *aacC1* gene conferring resistance to gentamicin. The A1arp+/vlsE- clone was generated by transforming the A1arp+/vlsE- strain with pBSV2 containing *vlsE* and *pncA* (pBSV2-vlsE) along with their native promoter sequences. This plasmid was constructed by first cloning *pncA* into the pBSV2 (Stewart et al., 2001) shuttle vector (at Ncol and Fsel restriction sites) that harbors a flaB promoter-driven kanamycin gene (pBSV2-pncA). Next, the *vlsE* gene along with its upstream 93 bp region was amplified using primers containing KpnI and XbaI restriction sites for cloning into above plasmid construct. An additional strain A1arp+/vlsE- pBSV2-pncA was also constructed by transforming A1arp+/vlsE- with pBSV2-pncA. All constructed plasmids were confirmed via PCR and DNA sequencing using primers shown in Table S5.

**B. burgdorferi transformation**

*B. burgdorferi* B31-A1 cells were electroporated and cultured as previously described (Bankhead and Chaconas, 2007). DNA from culture-positive wells was extracted using a Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI), and used for PCR analysis to confirm the presence of the antibiotic-resistance gene and the presence or absence of *arp* and *vlsE* (Figure S4). Naturally occurring plasmid content for each verified transformant was determined by PCR (Figure S3) using plasmid-specific primers as previously described (Bunikis et al., 2011).

**Production of recombinant Arp**

A 941 bp region (from coordinates 250 to 1465) of lp28-1 was PCR amplified (Table S5) and cloned into the pET15b expression vector (Novagen, Madison WI) to introduce a 6X His-tag at the N terminus of Arp. The resulting plasmid was introduced into *E. coli* Rosetta (DE3) pLysS Competent Cells (Novagen). His-tagged Arp expression was induced by adding 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside, Novagen) to the culture. The protein was harvested and purified using the Ni-NTA Purification System (Thermo-fisher Scientific, Waltham, MA) under native conditions to preserve the three-dimensional structure of the protein. The purified protein content in each fraction was determined by Coomassie Plus Assay kit (Thermo Scientific, Rockford, IL).

**Production of Arp-specific antibodies**

Anti-Arp antibodies were raised in C3H mice (Jackson Laboratory, Bar Harbor, ME) by inoculating the mice with purified recombinant Arp as a water-in-oil emulsion formulation. A 0.5 mL volume of aqueous Arp antigen was emulsified in 0.5 mL of Titermax Gold (Sigma Aldrich, St. Louis, MO) adjuvant using a two-syringe, 3-way stopcock method. Each mouse was inoculated subcutaneously with 100 μl (36 μg protein) of the emulsion in four divided doses at four different sites. A booster dose of Arp antigen (total 12 μg) was administered in exactly in the same manner 28 days after the initial inoculation. Blood was collected (through cardiac puncture) after 2 weeks of booster dose and centrifuged at 6,000xg for 12 minutes at 4°C to separate serum from blood cellular components. Anti-Arp antibodies were isolated from serum using Melon Gel IgG Purification kit (Thermo Scientific).

**Generation of antiseras against A1arp+/vlsE-, A1arp+/vlsE-, and A1arp+/vlsE- clones**

Antisera was raised in C3H mice (C3H/HeJ, The Jackson Laboratory) for animal challenge experiments and western blot analysis, against A1arp+/vlsE-, A1arp+/vlsE-, and A1arp+/vlsE- clones. Because these clones either lack VlsE (A1arp+/vlsE- and A1arp+/vlsE-) or possess a non-variable VlsE (A1arp+/vlsE-), spirochetes are cleared within three weeks by immunocompetent mice. To generate a better immune response, C3H mice were infected via subcutaneous needle inoculation with a higher dose of spirochetes (10^7-10^8 total organisms per mouse), and the mice were re-inoculated every two weeks for a total of three inoculations. Blood was collected after two weeks of the last infection and serum was separated by centrifugation. Sera were pooled from four to five mice for use in various subsequent experiments.
Membrane proteins were extracted from A1

Tissue collection for spirochete detection

Western blot analysis

Extraction of membrane proteins

Flow Cytometry Assay

For flow cytometry experiments, approximately $10^7$ spirochetes from each strain were incubated in 1% paraformaldehyde for 10 minutes to minimize damage to spirochetes in multiple washing steps down stream. The spirochetes were subsequently washed with and then resuspended in HEPES-buffered Tyrode’s Solution (HTS) (Boston Bioproducts, Ashland, MA). The bacterial suspension was incubated with anti-Arp or anti-VlsE antibodies (1:50 fold dilution) for one hour at 4°C. After washing, the pellet was suspended in Triton X-114 detergent (2% vol/vol in PBS), and then incubated overnight at 4°C with rocking to solubilize the membrane lipoproteins in the detergent. The insoluble material containing protoplasmic cylinders were pelleted by centrifugation at 15000 x g for 30 min at 4°C. The supernatant was removed, and the pellet discarded. To phase separate the lipoprotein-containing detergent phase from the aqueous phase, the supernatant was incubated at 37°C for 15 min, followed by centrifugation at 15000 x g at room temperature for 10 minutes. The detergent phase was carefully removed, and both the detergent phase as well as the aqueous phase were washed three times in PBS and Triton X-114 (2% final concentration in aqueous phase fraction), respectively. Proteins in the detergent and aqueous phases were precipitated by cold acetone (−20°C) and pelleted by centrifugation. The supernatant was decanted, and the pellet was suspended in 1x Laemmli sample buffer (Bio-Rad Laboratories). The protein content was determined by the RC DC Protein Assay Kit II (Bio-Rad Laboratories). The proteins were run on Any Kd Mini-ProteanTGX precast polyacrylamide gels (Bio-Rad Laboratories) for staining with Oriole Fluorescent Gel stain (Bio-Rad Laboratories). To probe for membrane localization of Arp and VlsE in detergent phase, immunoblotting was carried out using anti-Arp and Anti-VlsE antibodies (Rockland, Limerick, PA) to ensure that detergent phase contained only membrane proteins and aqueous phase only water-soluble proteins, anti-BosR antibodies (General Bioscience, Brisbane, CA) were used to confirm the presence of BosR (cytosolic protein) in the aqueous fraction, but absence in the detergent fraction.
**Immunofluorescence Assay**

Immunofluorescence was carried out with some modifications of previously described protocols (Bryksin et al., 2010; Mulay et al., 2007; Zhang et al., 2011). Spirochetes were grown to late log-phase, centrifuged to remove the medium and washed three times in HEPES-buffered Tyrode’s Solution (HTS) (Boston Bioproducts) to remove residual medium components including serum. The cell pellet containing approximately $10^7$ spirochetes was re-suspended in HTS. The spirochetes were incubated in primary antibodies (1:50 fold dilution) for one hour at $35^\circ C$. The antibodies used were mouse anti-Arp and anti-A1arp/vlsE antisera, and rabbit anti-FlaB antibodies (Rockland). After incubation, the cells were washed with HTS, and then resuspended in the same buffer. Ten microliters of this suspension were deposited on positively charged glass slides (Probe On Pulse, Fisher Scientific), dried, and free sites on the slide were blocked by casein-based blocking buffer (casein in PBS buffer with Tween 20, Abcam). This was followed by incubating the spirochetes in either Alexa Fluor 647-labeled donkey anti-mouse IgG (ThermoFisher Scientific, Rockford, IL) or donkey anti-rabbit IgG (Abcam, Cambridge, MA) antibodies (1:500 fold dilution in blocking buffer), depending upon the species of origin of the primary antibody. The slides were again washed in PBS, followed by fixation of spirochetes in 4% paraformaldehyde for 10 min. The slides were finally mounted with coverslips, using Prolong Gold Antifade mountant containing DAPI (ThermoFisher Scientific). The bacteria were imaged with appropriate wavelength laser light using Leica SP8 confocal microscope (Leica Microsystems Inc., Deerfield, IL).

To ascertain that the spirochete proteins of our interest are targeted by the primary antibodies only when located on the cell surface, we used antibody anti-FlaB antibodies (targets periplasmic FlaB) as described above. That periplasmic protein FlaB can be accessed by anti-FlaB antibodies only after membrane is permeabilized, five microliters of bacteria were also deposited on positively charged slides, dried and then fixed in methanol for 20 minutes. The slides were washed in PBS (pH 7.4) and free sites were blocked by the casein-based blocking buffer (Abcam). The spirochetes were incubated with anti-FlaB antibody (1:50 dilution) for one hour, then washed in PBS before being incubating in Alexa Fluor 647-labeled donkey anti-mouse IgG (ThermoFisher Scientific, Rockford, IL) at room temperature for one hour. The slides were again washed in PBS and were mounted with coverslips, using Prolong Gold Antifade mountant containing DAPI (ThermoFisher Scientific) and visualized using confocal microscopy as above.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical parameters including statistical significance is indicated in the table footnote. To test overall statistically significant difference across all treatment groups, Fisher’s exact test was used, while Bonferroni correction was used for differences between groups. $p < 0.05$ was considered statistically significant difference.

**DATA AND CODE AVAILABILITY**

The published article contains most of the data; a request for any raw data can be made to the lead contact.