Tick-host-Borrelia interaction

Implications for host immunity and vaccination strategies

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Chapter 9

Variable major proteins as targets for specific antibodies against *Borrelia miyamotoi*

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**ABSTRACT**

*Borrelia miyamotoi* is a relapsing fever spirochete in Ixodes ticks that has been recently identified as a human pathogen causing hard tick-borne relapsing fever (HTBRF) across the Northern Hemisphere. No validated serologic test exists, and current serologic assays have low sensitivity in early HTBRF. To examine the humoral immune response against *B. miyamotoi*, we infected C3H/HeN mice with *B. miyamotoi* strain LB-2001 expressing variable small protein 1 (Vsp1) and demonstrated that spirochetemia was cleared after 3 d, coinciding with anti-Vsp1 IgM production. Clearance was also observed after passive transfer of immune sera to infected SCID mice. Next, we showed that anti-Vsp1 IgG eliminates Vsp1-expressing *B. miyamotoi*, selecting for spirochetes expressing a variable large protein (VlpC2) resistant to anti-Vsp1. The viability of Asian isolate *B. miyamotoi* HT31, expressing Vlp15/16 and Vlp18, was also unaffected by anti-Vsp1. Finally, in nine HTBRF patients, we demonstrated IgM reactivity to Vsp1 in two and against Vlp15/16 in four ~1 wk after these patients tested positive for *B. miyamotoi* by PCR. Our data show that *B. miyamotoi* is able to express various variable major proteins (Vmps) to evade humoral immunity and that Vmps are antigenic in humans. We propose that serologic tests based on Vmps are of additional value in diagnosing HTBRF.
*B. miyamotoi* is a tick-borne relapsing fever (TBRF) spirochete that is present in several *Ixodes* tick species across the Northern Hemisphere (1, 2). Although its existence has been recognized since 1994, *B. miyamotoi*’s potential to infect humans was not discovered until 2011, when patients suffering from a nonspecific (viral-like) febrile illness were found to be infected with *B. miyamotoi* (3). Since then, various reports have described clinical cases of *B. miyamotoi*-infected patients in the United States and Japan, confirming the clinical presentation of high fever, chills, severe headache, myalgia, and/or arthralgia (4–7). Chronic CNS infections have been described in two immunocompromised patients receiving B cell–depleting therapy in the United States and Europe (8, 9). The disease entity caused by *B. miyamotoi* has been termed *B. miyamotoi* disease (7) or hard TBRF (HTBRF) (10), of which we use the latter description throughout this article. Recently, *B. miyamotoi* has been successfully propagated in culture using two different culture media, both of which use modified Kelly–Pettenkofer (MKP) medium with addition of bovine or human serum (11, 12). Although peak concentrations of *B. miyamotoi* remain relatively low, these methods, combined with whole-genome sequencing (13), may contribute to the discovery of new serological markers and aid the understanding of the disease pathogenesis. Currently, HTBRF is diagnosed by PCR on blood during acute illness, whereas serodiagnosis has been performed using the glycerophosphodiester phosphodiesterase (GlpQ) Ag, which is present in TBRF Borrelia species, but not in *B. burgdorferi* s.l. (7, 14–17). A recent study showed that 11% of HTBRF patients had IgM reactivity in a rGlpQ enzyme immunoassay upon presentation, whereas 64% demonstrated IgM seroconversion to GlpQ in convalescent sera (7). These findings underscore the need for additional early seromarkers to support a diagnosis of *B. miyamotoi* infection at disease onset or after antibiotic treatment, when quantitative PCR might be negative. A study in the Netherlands revealed a higher prevalence of GlpQ Abs among forestry workers, Lyme disease patients, and those suspected to have human granulocytic anaplasmosis, suggesting they had been infected with *B. miyamotoi* (17). However, there have not been any studies investigating which *B. miyamotoi* proteins are most antigenic. *B. miyamotoi* was reported to express *vsp* genes two decades ago (18), and a recent study confirmed the presence of *B. miyamotoi* genes coding for variable major proteins (Vmps), also revealing several variable large proteins (Vlps) (19). TBRF spirochetes are able to switch serotypes by nonreciprocal gene transfer of these immunogenic Vmps, thereby evading the host Ab response and enabling relapses to occur (20–24). This system has been extensively studied in *Borrelia hermsii*, where 59 genes coding for Vmps have been identified on archival plasmids in nonexpression loci, consisting of immunogenic variable small proteins (Vsps, ~22 kDa) and Vlps
The dimeric Vsps have a different protein structure compared with the monomeric Vlps and only a remote evolutionary relationship (26, 29). At each moment in time, only one of these serotype-defining Vmps is expressed by a spirochete, namely when this vsp/vlp gene has been copied into the expression site that is located on a linear plasmid. However, populations can consist of several serotypes, and serotype switching can also occur spontaneously in a small fraction of spirochetes, with an estimated frequency of $10^3$ to $10^4$ per spirochete per generation (30). Thus, infected hosts will clear TBRF spirochetes by IgM directed against one or a few dominant Vmps, leaving outlier spirochetes that express different Vmps to replicate and cause a relapse of spirochtemia (30, 31). *Borrelia turicatae* Vsps have been described to have a conserved core and a variable exposed dome, explaining why IgM raised against one Vsp is less likely to bind another, and they have been shown to exert different tissue tropisms (27, 32–35). For *B. miyamotoi*, this system is yet to be unraveled. Based on the presence of vmp genes in *B. miyamotoi* and the involvement of Vmps in TBRF pathogenesis, it could be expected that *B. miyamotoi* Vmps are variably expressed, immunogenic, and involved in immune evasion. In this study we experimentally infected mice with in vitro–cultured *B. miyamotoi* LB-2001, a tick isolate from Connecticut, and used the evolving humoral immune response to identify novel *B. miyamotoi* Ags expressed in early infection. We identified Vsp1 as a dominant antigenic target and show that Abs to this Ag are capable of eliminating most spirochetes in *B. miyamotoi* LB-2001–infected SCID mice after passive transfer and in cultured *B. miyamotoi* LB-2001 in vitro. Surviving spirochetes expressed a different Vmp and were resistant to anti-Vsp1 Ab-mediated killing. Finally, we show that Vmp-specific Abs can be detected in HTBRF patient sera, providing insight into HTBRF pathogenesis and revealing *B. miyamotoi* Vmps as additional early serodiagnostic markers.

**MATERIALS AND METHODS**

**Infection, passive transfer, and immunizations with Borrelia lysates**

Stocks of a P4 passage of *B. miyamotoi* LB-2001 were cultured in MKP-F medium from 280°C glycerol stocks (12). Seven-day cultures of fifth passage spirochetes were counted using a Petroff–Hauser counting chamber and 6- to 8-wk-old female C3H/HeN mice (Charles River Laboratories) were infected by i.p. injection with $10^7$ spirochetes in 200 μl PBS. Five- to seven-week-old CB17 Fox Chase SCID mice were similarly infected using $10^6$ spirochetes in PBS. Passive transfer was performed by i.p. injection of 250 μl plasma from C3H/HeN mice infected 5 or 14 d earlier, after syringe
filtering and confirming the absence of spirochetes by dark-field microscopy. *B. miyamotoi* LB-2001 and *B. burgdorferi* 297 were cultured for 7 d in MKP-F medium or Barbour–Stoenner–Kelly medium (Sigma-Aldrich, St. Louis, MO) at 33˚C, washed four times in sterile PBS, and heat inactivated at 56˚C for 20 min, followed by sonication (six times at 15 s). Groups of five C3H/HeN mice were immunized with 5 μg of either lysate emulsified with complete Freund’s adjuvant (first immunization) or incomplete Freund’s adjuvant (two boosters after 2 and 4 wk), and serum was obtained after 6 wk. Passive transfer of these antisera was performed similar to the method used for naturally immune sera. For analysis of Vsp1 expression in culture, *B. miyamotoi* LB-2001 was passaged 50 times, and glycerol stocks of P5, P10, P20, P30, P40, and P50 cultures were inoculated in triplicate at 33˚C from -80˚C for 7 d until a mean concentration of 1.3 x 10^7 /ml to 2.6 x 10^7 /ml, followed by generation of lysates as described above, which were run on 4–20% SDS gel and stained by Coomassie blue.

**Protein identification**

*B. miyamotoi* LB-2001 was cultured in MKP-F medium at 33˚C from a P4 -80˚C glycerol stock for 7 d, after which spirochetes were washed four times by centrifuging (10,000 x g for 7 min) and resuspended in sterile PBS. Suspensions were heat inactivated for 20 min in a water bath at 56˚C and sonicated six times for 15 s (Branson Ultrasonics, Danbury, CT). A total of 7.5 μg was loaded in a mini-protean 4–20% SDS gel (Bio-Rad, Hercules, CA) together with *B. burgdorferi* 297 lysate (cultured for 7 d in Barbour–Stoenner–Kelly medium). Gel bands ∼23–25, ∼37–39, and ∼59– 63 kDa were sent to the Yale School of Medicine Keck proteomics laboratory for trypsin digestion followed by liquid chromatography–tandem mass spectrometry on an LTQ Orbitrap (Thermo Scientific, Waltham, MA) followed by a BLAST on the MASCOT database. A 37-kDa band from *B. miyamotoi* HT31 and a 35-kDa band from *B. miyamotoi* LB-2001 after Vsp1 IgG challenge were analyzed by York University Department of Biology, where tryptic peptides were analyzed by MALDI–mass spectrometry and tandem mass spectrometry using a Bruker Ultraflex III MALDI-TOF/TOF.

**In silico analysis**

The amino acid sequences of *B. miyamotoi* (GenBank AGS80212.1) and *B. turicatae* Vsp1 (GenBank AAB65089.1) were aligned using AlignX software (Invitrogen, Carlsbad, CA) and visualized using BoxShade 3.2 Web-based software (ExPASy, Swiss Institute of Bioinformatics). The signal sequence was predicted using a model for spirochetal lipoprotein signal sequence prediction (36). Protein structure prediction of *B. miyamotoi* Vsp1 was performed using the online homology modeling server Protein Homology/analogY Recognition Engine version 2.0 (Phyre2; Imperial College,
London, U.K.) and visualized using the PyMOL molecular graphics system, version 1.7.4 (Schrödinger). Three annotated crystal structures of *B. burgdorferi* OspC and one *B. turicatae* Vsp1 structure yielded 100% confident structural homologies of residues 45–211 (44% identical, Protein Data Bank [PDB] 1F1M, template d1f1ma), 45–214 (42% identical, PDB 1G5Z, template d1g5za), 45–210 (47% identical, PDB 1GGQ, template d1ggqqa), and 51–208 (57% identical, PDB 1YJG, template c1yjgE), respectively. The predicted Vsp1 monomer has been aligned to the *B. turicatae* Vsp1 dimer crystal structure (PDB 2GA0) to predict its dimeric structure.

**Recombinant protein generation**

DNA was extracted from *B. miyamotoi* LB-2001 and HT31 (cultured to passage 5) using a blood and tissue kit (Qiagen). A PCR was performed with Phusion high-fidelity mix (New England Biolabs, Ipswich, MA) and forward primer 5’-AAAAGCTAGCTGTGGAAGTGGGG-3’, and using reverse primer 5’-AAAACTCGAGTGAAGATTGACCAGC-3’ for LB- 2001 and HT31. After 98°C for 30 s, 25 cycles of 98°C for 10 s, 62°C for 30 s, and 72°C for 30 s were performed and PCR products were ligated into the pet21b vector using Nhe1 and Xho1 restriction sites. In both *B. miyamotoi* isolates an identical Vsp1 sequence was identified, with consistently two base point mutations compared with the annotated sequence identified by whole-genome sequencing (GenBank KF031441): nucleotides 223 and 287 (both guanine instead of adenosine), leading to a N75→D75 substitution and a D96→G96 substitution, respectively, compared with the annotated sequence. The LB-2001 construct was transformed into BL21 (DE3) cells (Novagen, Madison, WI) and 300 ml Luria–Bertani/ampicillin culture was induced with isopropyl b-D-thiogalactopyranoside at OD 0.5–0.8 and incubated overnight at 30°C. Cells were resuspended in sterile PBS, and 1 mg/ml lysozyme (Sigma-Aldrich) was added and samples were sonicated six times for 15 s. Next, 1% Triton X-100 was added and samples were incubated for 30 min at 4°C, centrifuged, and filtered using a 0.22-μm membrane. EDTA-free protease inhibitor (Roche) was added, and samples were run over Ni-NTA columns using manufacturer instructions and eluted using 300 mM imidazole. Pooled fractions were dialyzed four times using 9-kDa Amicon ultra centrifugal filters (Merck Millipore). rVlp15/16 (WP_025444482.1) was similarly generated using primers 5’-AAAAGCTAGCTGTGTAATAATGGAGGAGGGG-3’ and 5’-AAAAACTCGAGCTCTGCTGTGGAAGTGGGGG-3’; rVlp18 (WP_025444235.1) was cloned using 5’-AAAAGCTAGCTGTGTAATAATGGAGGAGGGG-3’ and 5’-AAAAACTCGAGCTCTGCTGTGTAATAATGGAGGAGGGG-3’, both from *B. miyamotoi* HT31 DNA. Protein and Ab concentrations were determined using a DC protein assay (Bio-Rad).
**ELISAs**

To measure IgM and IgG directed against recombinant proteins, high-binding half-surface plates (Greiner Bio-One) were coated overnight at 4°C with 50 nM recombinant protein. Next, plates were washed in PBS–Tween 20 (0.05%) and incubated for 2 h with blocking buffer (PBS plus 1% BSA) at room temperature. Plates were subsequently washed and incubated for 1 h at room temperature with 1:1000 C3H/HeN mouse sera in blocking buffer. Plates were washed and incubated for 30 min with 1:1000 antimouse IgG-HRP (Cell Signaling Technology, Danvers, MA) or 1:2000 anti-mouse IgM (Southern Biotech, Birmingham, AL) in blocking buffer. Finally, plates were washed and developed using TMB substrate, and absorbance was read at 450 nm (Bio-Tek, Winooski, VT). In the case of rabbit anti-Vsp1 antisera, sera were diluted 1:10,000, and 1:4,000 anti-rabbit IgG-HRP (Cell Signaling Technology) was used as a secondary Ab. IgM against *B. miyamotoi* LB-2001 or *B. burgdorferi* 297 lysates was similarly performed using 1 μg/ml lysate in PBS as coating Ag, 1:100 diluted mouse sera as primary Ab, and 1:2000 anti-mouse IgM (Life Technologies, Carlsbad, CA) as a secondary Ab.

**Western blots**

Western blots were performed by loading 3 μg *Borrelia* lysate on miniprotean 4–20% SDS gels (Bio-Rad), blotting to polyvinylidene difluoride membranes, and blocking overnight in blocking buffer (TBS with 0.05% Tween 20 and 5% skim milk powder) at 4°C. The next day, blots were incubated for 1 h in mouse serum diluted 1:100 (5 and 7 d postinfection) or 1:200 (14 d postinfection) in TBST with 2.5% skim milk powder. Membranes were washed three times in TBST and incubated 1:10,000 with donkey/goat anti-mouse IgM/IgG 800CW (LI-COR Biosciences, Lincoln, NE) for 30 min, followed by three washes and developed on a LI-COR Odyssey infrared imager. Western blots comparing *B. miyamotoi* isolates LB-2001 and HT31 were performed on 250 ng lysates made from P4 passages from a -80°C stock cultured for 7 d at 33°C. Membranes were incubated with 1:500 diluted sera from four individual mice 14 d postinfection, with anti-mouse IgG-HRP (Cell Signaling Technology, Beverly, MA) as a secondary Ab, and developed on an ImageQuant LAS 4000 (GE Healthcare Biosciences, Pittsburgh, PA). Western blots on recombinant proteins were performed by electrophoresis of 500 ng recombinant proteins, incubation with 1:1000 pooled sera for 1 h at room temperature, and 1:10,000 anti-mouse IgM-HRP (Southern Biotech) or anti-mouse IgG-HRP (Cell Signaling Technology) for 30 min. Western blots on *B. miyamotoi* cultured after Vsp1/OVA Ab challenge were performed using 500 ng lysates and 1:4,000 rabbit anti-Vsp1, followed by 1:10,000 anti-rabbit IgG-HRP (Cell Signaling Technology), or using 1:1,000 pooled mouse sera (n = 4) from C3H/
HeN mice (naive/5 d after *B. miyamotoi* infection/ 14 d postinfection) and 1:10,000 HRP-linked anti-mouse IgM or IgG. Western blots on human sera were performed similarly, using 500 ng recombinant proteins and incubation in human sera diluted 1:500 followed by 1:10,000 anti-human IgM-HRP (Southern Biotech) or IgG-HRP (BioRad). Human sera were collected from Russian HTBRF patients (3, 37) where diagnosis was confirmed by PCR and sequencing. All HTBRF patients developed a fever >38°C axillary temperature. No relapses were reported in these patients, as all patients were antibiotically treated shortly after disease onset. Sera from Russian patients hospitalized for 8–15 d for infectious disease with a typical erythema migrans (EM) and a history of tick bite were used. Sera from healthy blood donors were used as controls.

**Dark-field spirochete counts**

Blood samples (30 μl) were obtained by tail nick in Microvette lithium-heparin plasma tubes (Sarstedt, Numbrecht, Germany). Blood (2.5 μl) was added to 47.5 μl SideStep lysis and stabilization buffer (Agilent Technologies, Santa Clara, CA), and the rest of the blood was centrifuged at 500 x g for 5 min as described elsewhere to obtain plasma (38). Plasma samples were counted under dark-field microscope using a Petroff–Hauser counting chamber, using a 1-mm² counting square according to the manufacturer’s instructions. When no spirochetes were observed, eight additional squares of 1 mm² were counted to obtain a detection limit of 5.555 spirochetes/ml, and total spirochete concentrations were calculated based on 9-mm² observed surface, or given the detection limit when negative.

**Anti-Vsp1 Ab generation and killing assays**

Two rabbits were immunized with 200 μg rVsp1 s.c. in complete Freund’s adjuvant (t = 0) and incomplete Freund’s adjuvant (t = 14, 28, and 56 d) (Eurogentec, Liège, Belgium) and sacrificed at t = 65 d. Final pooled sera were purified using a Zeba spin desalting column (Life Technologies) and a Melon gel IgG spin purification kit (Life Technologies) and filter sterilized using a 0.22-μm filter. A titration killing assay was performed using various concentrations of polyclonal anti-Vsp1 IgG (0–1000 μg/ml) in 5 μl PBS added to V-shaped sterile microtiter plates. *B. miyamotoi* LB-2001 at a concentration of 3 x 10⁶/ml was added (32.5 μl/well) together with 12.5 μl pooled normal human serum (NHS) at a final concentration of 25%. As a control, heat-inactivated human serum (NHS incubated at 56°C for 30 min) was added instead at the same concentration. Wells were resuspended and the plate was incubated at 37°C for 1 h followed by blinded dark-field microscopy scoring of 100 spirochetes per well (12), using immobilization as a marker for spirochete mortality (39–42). *B.*
miyamotoi HT31 and B. burgdorferi 297 P4 strains cultured from -80°C stocks were equalized to $3 \times 10^6$ /ml using MKP-F medium and subjected to a final concentration of 100 μg/ml anti-Vsp1 IgG. The remaining 45 μl per well in the B. miyamotoi LB-2001 suspensions subjected to anti-Vsp1 or anti-OVA IgG (43) was inoculated into 1.6 ml MKP-F in 2 ml screwcap tubes, and after 1 wk at 33°C they were passaged at the same concentrations into 7 ml MKP-F followed by two 7-d passages at 33°C and storage at -80°C in 4% glycerol-peptone, from which they were cultured to generate lysates.

**PCR for B. miyamotoi LB-2001 expression site on Vsp1 and VlpC2**

DNA was extracted from a P3 passage of B. miyamotoi LB-2001 isolates 1 (expressing Vsp1) and 2 (anti-Vsp1 exposed) using a DNeasy blood and tissue kit (Qiagen). We designed a forward primer based on the “-35” promoter element of B. miyamotoi LB-2001, which was located upstream of the annotated Vsp1 gene as described above: 5’-GAATTTGAAAAGTAAGATTCTTGCAC-3’. To identify the sequence for the B. miyamotoi LB-2001 35-kDa band, which is dominantly expressed by a population surviving anti-Vsp1 IgG challenge, PCR was performed with Phusion high-fidelity PCR master mix (New England Biolabs) using the forward “-35” promoter element primer and reverse primer 5’-TTATTTACCTTTTAGCTTCAGAGGTCTTATTAT-3’, which was based on the gene coding for a protein to which three peptides of the mass spectrometry of this band had matched, that is, B. miyamotoi Fr64b Vlp5. PCR conditions were 98°C for 30 s, followed by 30 cycles of 98°C for 10 s, 60°C for 30 s, and 72°C for 30 s, and a final 10-min extension step at 72°C. The PCR product was purified and sequenced, and a partial gene was annotated sharing 99% identity with the B. miyamotoi gene *vlpc2*. Furthermore, a reverse primer 5’-GCACTTTTTCATGAGCATC-3’ was designed to identify Vsp1 in the expression site, and reverse primer 5’-TACCTGCTTCACCATCAC-3’ (with a similar melting temperature) was designed based on the *vlpc2* sequence. A PCR was performed using Phusion high-fidelity PCR master mix using 1 μl DNA, 2.5 μl of both primers (10 mM), 19 μl H2O, and 25 μl Phusion master mix. A 98°C for 30 s denaturation was followed by 30 cycles of 98°C for 10 s, 57.5°C for 30 s, and 72°C for 15 s, followed by 10 min at 72°C.

**Ethics statement**

Animals were housed and handled under the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal research protocols were approved by Yale University’s Institutional Animal Care and Use Committee (protocol no. 2014-07941) and by the Academic Medical Center’s Ethical Committee for Animal Research (protocol no. DIX103058).
Statistical analysis

One-way ANOVA was performed on ELISA results using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). When \( p < 0.05 \), individual columns were compared using a Student t test. Killing assay motility percentages were compared with the control (PBS or anti-OVA) using a Mann–Whitney U test. A \( \chi^2 \) test was performed in a case when all replicates were similar.
RESULTS

*B. miyamotoi LB-2001 expresses Vsp1, which induces a dominant Ab response in C3H/HeN mice*

We set up a mouse model where $10^7$ *B. miyamotoi* LB-2001 spirochetes (passaged up to four times in MKP-F medium) were inoculated in 200 μl PBS i.p. into C3H/HeN mice. Plasma was examined by dark-field microscopy for spirochetes daily, and a peak in spirochetemia was observed after 2 d, followed by lowgrade spirochetemia in three of eight mice after 5 or 6 d, one of which was at the detection limit at day 5 (Fig. 1A). In most mice, however, the pathogen remained below the detection limit from day 3, coinciding with a rise in *B. miyamotoi*–specific IgM (Fig. 1B). Infected mice developed detectable anti–*B. miyamotoi* IgG at day 5 that increased at day 14 after inoculation (Fig. 1C). Although *B. miyamotoi* and *B. burgdorferi* s.l. are spirochetes of the same genus in the same tick vector, they have a different protein expression profile (Fig. 1D). We performed mass spectrometry on the three dominant protein bands corresponding to molecular masses of ~60, ~39, and 23 kDa in *B. miyamotoi* LB-2001 lysate. This revealed that the ~60-kDa band was comprised of mostly 30S ribosomal protein S1 (GenBank WP_020954521.1, predicted molecular mass of 62.6 kDa) and GroEL (WP_020955008.1, 58.9 kDa), whereas the ~39-kDa (reactive) band contained GlpQ (WP_020954631.1, 38.7 kDa) in the highest abundancy, followed by N-acetylglucosamine-6-phosphate deacetylase (WP_020955113.1, 39.4 kDa) and flagellin (WP_020954538.1, 35.4 kDa) (Fig. 1D). The prominent 23-kDa band was found to contain Vsp1 (AGS80212.1; 23.3 kDa, Supplemental Fig. 1A) and co-chaperone GrpE (WP_020954892.1; 21.3 kDa). Western blots using 5- and 14-d infected mouse sera showed that the IgM and IgG responses were mainly directed against the 23-kDa *B. miyamotoi* protein band, and did not react with any 23-kDa protein in *B. burgdorferi* lysate that was run in adjacent lanes as a control (Fig. 1E, 1F). Because of its dominant expression, and because VMPs are immunogenic in other TBRF species, we hypothesized that Vsp1 was responsible for the reactivity against the 23-kDa protein.
**Figure 1.** *Borrelia miyamotoi* infection induces a robust Ab response against a 23 kDa protein. 
*B. miyamotoi* infection induces a robust Ab response against a 23-kDa protein. (A) Dark-field microscopy on plasma from eight C3H/HeN mice after i.p. inoculation with *B. miyamotoi* LB2001 spirochetes. Horizontal lines represent mean concentrations, negatives represent the detection limit. (B) ELISA detecting IgM against *B. miyamotoi* LB-2001 whole-cell lysate. (C) IgG response to infection demonstrated by ELISA using sera from mice infected 5 or 14 d postinfection. (D) SDS-PAGE of *B. miyamotoi* whole-cell lysate stained by Coomassie brilliant blue, revealing abundant protein bands at ~23, ~39, and ~60 kDa. These bands were analyzed by liquid chromatography–tandem mass spectrometry, and the most abundant proteins are depicted on the left. For comparison, *B. burgdorferi* lysate (B.b 297) was run in the right lane. (E and F) Western blot analysis of nitrocellulose strips loaded with *B. miyamotoi* LB-2001 (Bm) and *B. burgdorferi* 297 (Bb) whole-cell lysates, detecting IgM in sera of four mice 5 d after *B. miyamotoi* infection (E) or IgG 14 d postinfection in seven mice (F). Protein size in kDa is depicted on the left. Error bars illustrate mean ± SEM. *p < 0.05, **p < 0.01.

**Vsp1 in silico analysis**

Vsp1 appeared to be the dominantly expressed Vmp in this *B. miyamotoi* population, with its gene being located on a 26-kb plasmid fragment behind a σ70-type prokaryotic promoter. A comparison of the upstream sequence of the expressed Vsp1 sequence with the *vsp/vlp* promoter region of *B. turicatae* revealed homology at the “-35” element, which was flanked by AT-rich inverted repeats, as well as an identical ribosomal binding site (Fig. 2A). Next, we were interested whether the Vsp1 protein structure was similar to the previously described *B. turicatae* Vsp1. First, we aligned the Vsp1 amino acid sequence with that of *B. turicatae* Vsp1, the only Vsp for which a crystal structure has been described (Research Collaboratory for Structural Bioinformatics Protein Data Bank accession no. 2GA0_A) (32). In silico analyses revealed 55% identity and 70% consensus positions with the amino acid sequence of *B. turicatae* Vsp1 (Fig. 2B). A homology model with 100% confidence of the *B. miyamotoi* Vsp1 structure was generated in silico using the Phyre2 Web server (44) (Fig. 2C). The predicted structure of *B. miyamotoi* Vsp1 consists of four α helices forming a bundle with two small β-strands pointing outwards, and hydrophobic residues line the core of the α-helical bundle.
Figure 2. *Borrelia miyamotoi* Vsp1 promoter region and predicted protein structure.

(A) Alignment of the promoter regions of the *B. miyamotoi* and *B. turicatae* expression sites, based on *B. miyamotoi* Vsp1 (KF031441.1) and *B. turicatae* VspB (AF049852) sequences. AT-rich sequences surround the “-35” promoter element. RBS, ribosomal binding site; Start, start codon. (B) Alignment of *B. miyamotoi* Vsp1 and *B. turicatae* Vsp1 amino acid sequences, including their respective signal sequences, conserved amino acids (gray background), and identical residues (black background). (C) Structure prediction based on annotated crystal structures of *B. turicatae* Vsp1 and *B. burgdorferi* OspC using the Phyre2 Web portal. Vsp1 and OspC are known to form dimers, and in this figure a predicted monomer (dark gray) has been aligned to a *B. turicatae* Vsp1 dimer crystal structure (PDB 2GA0), consisting of two *B. miyamotoi* Vsp1 monomers (light and dark gray, respectively).

Verification of Vsp1 Abs in mice

To verify our hypothesis that Vsp1 was one of the immunodominant *B. miyamotoi* proteins recognized by *B. miyamotoi*-infected mouse sera, we generated a recombinant His-tagged *B. miyamotoi* LB-2001 Vsp1 (rVsp1), without its predicted 24-aa signal sequence to enhance solubility (Fig. 3A). ELISAs using rVsp1 as an Ag were performed on sera from *B. miyamotoi* LB-2001–infected mice. Indeed, a rise in anti-Vsp1 IgM was observed in infected mice from day 3, with a maximum level at day 5 postinfection (Fig. 3B). Anti-Vsp1 IgM levels decreased at day 14 postinfection (Fig. 3C), at which time anti-Vsp1 IgG levels were >3 SD higher than those of naive control mice (Fig. 3D). A Western blot using pooled sera from infected C3H/HeN mice further confirmed the presence of IgM and IgG reacting with rVsp1 (Fig. 3E). Of note, C3H/HeN mice developed more robust Ab responses against Vsp1 compared with GlpQ, which is currently used as a seromarker for human infection (Fig. 3F).
Figure 3. A rapid Vsp1 specific IgM response is induced upon *B. miyamotoi* infection. 

(A) rVsp1 loaded onto a 4–20% SDS-page gel. M, protein marker (size in kDa on the left). 

(B–D) ELISAs were coated with rVsp1 and incubated with previously infected mouse plasma, detecting IgM (B and C) and IgG (D). Bars represent mean ± SEM. 

(E and F) Western blot and ELISA detecting IgM and IgG against GlpQ, Vsp1, or a negative control protein (tHRF) in mouse sera 5 or 14 d postinfection. For Western blot, pooled sera (n = 4) were used. For ELISA, individual mouse sera (5 d postinfection, n = 5; 14 d postinfection, n = 8) were used. 

(G) Three groups of three SCID mice were inoculated i.p. with either 10^3, 10^5, or 10^7 *B. miyamotoi* LB-2001 spirochetes, and plasma spirochete concentrations were measured by dark-field microscopy. 

(H) Dark-field microscopy in plasma of *B. miyamotoi*-infected SCID mice (n = 4/group) receiving an i.p. passive transfer of pooled sera at t = 8 d postinfection. Mice received 250 μl naive or 5- or 14-d immune C3H/HeN mouse sera. Error bars illustrate mean ± SEM, negatives represent the detection limit. **p < 0.01, ***p < 0.001.
Effects of passive immunization on B. miyamotoi spirochetemia in SCID mice

We next set out to investigate whether Abs present in serum 5 and 14 d postinfection, which included anti-Vsp1 IgM and IgG Abs, were sufficient to clear B. miyamotoi infection. To this end, we used SCID mice that develop persistent spirochetemia upon infection with B. miyamotoi (13, 45). We therefore performed a dose-finding experiment in which we i.p. inoculated SCID mice with $10^3$, $10^5$, or $10^7$ B. miyamotoi LB-2001 spirochetes. As expected, spirochetes were detected more rapidly using the highest inoculum, but all mice became spirochetemic within 6 d after inoculation and the levels plateaued at concentrations ranging from $10^7$ to $10^8$ spirochetes/ml (Fig. 3G). We subsequently infected groups of four SCID mice with $10^5$ B. miyamotoi spirochetes i.p., and after 8 d, 250 μl pooled serum from previously infected C3H/HeN mice was injected i.p. to assess its potential to clear spirochetemia. Tail bleeds were performed to check for B. miyamotoi concentrations in SCID mouse plasma. Whereas passive transfer of naive mouse sera did not show any effect on spirochetemia, 5- and 14-d immune sera were both successful in temporarily clearing B. miyamotoi (Fig. 3H). Interestingly, both sera were unable to permanently clear B. miyamotoi, and relapses were observed around day 14 and days 20–22 in mice treated with 5- and 14-d immune sera, respectively. Finally, we immunized mice with Vsp1-expressing B. miyamotoi lysates, yielding a strong anti-Vsp1 IgG response (Fig. 4A–C). Although the anti-Vsp1 IgG levels were higher than in serum of mice experimentally infected with B. miyamotoi LB-2001 14 d postinfection, passive transfer of immunized mouse sera to infected SCID mice did not result in more efficient clearance of spirochetemia (Fig. 4D). These data demonstrate that Abs against B. miyamotoi are involved in (partial) clearance in vivo. The observation that Vsp1 was the most dominant antigenic protein during natural infection and after immunization with B. miyamotoi lysate suggests a role for Vsp1-specific Abs.
Figure 4. Serum from mice immunized against Vsp1 serotype B. miyamotai temporarily clears B. miyamotai spirochetemia in SCID mice.

(A) Western blot of strips containing B. miyamotai LB-2001 (Bm) and B. burgdorferi 297 (Bb) lysate incubated with pooled antiserum from mice immunized with either B. burgdorferi 297 or B. miyamotai LB-2001 lysate. (B and C) B. miyamotai antiserum reacted with rVsp1 as shown by Western blot and ELISA, respectively. (D) Dark-field microscopy in plasma of B. miyamotai–infected SCID mice receiving passive transfer of pooled B. miyamotai antiserum, B. burgdorferi antiserum, or naive serum 8 d after initial infection. Error bars illustrate mean ± SEM. **p ≤ 0.01, ***p ≤ 0.001.
**Vsp1 Ab-mediated killing**

To confirm that Vsp1 Abs are involved in the elimination of *B. miyamotoi*, we generated anti-Vsp1 IgG and performed killing assays on cultured *B. miyamotoi* spirochetes. We generated polyclonal anti-Vsp1 IgG through immunization of two rabbits with rVsp1. Next, to confirm the stability of Vsp1 expression in vitro, we cultured *B. miyamotoi* for 50 wk using weekly passages, confirming stable expression after 50 passages (data not shown). Subsequently, we performed killing assays using increasing concentrations of pooled purified anti-Vsp1 IgG added to five replicate wells containing $10^5$ *B. miyamotoi* spirochetes in the presence of 25% human serum (NHS), and we determined viability after 1 h by assessing the percentage of motile spirochetes during dark-field examination of 100 spirochetes per well. This resulted in a nearly complete killing from 25 μg/ml Ab (Fig. 5A), with extensive bleb formation. Interestingly, when heat-inactivated human serum was used, thus eliminating complement activity, no bactericidal effect was observed using 25 μg/ml anti-Vsp1 IgG, indicating a role for complement in spirochete killing. However, killing was observed even in heat-inactivated human serum when a higher Ab dose (100 μg/ml) was administered, suggesting an additional complement-independent mechanism of Ab-mediated killing (Fig. 5A). Next, we examined whether Vsp1 is also expressed by a population of *B. miyamotoi* HT31, a tick isolate derived from Japan. Coomassie staining on spirochete lysates revealed that instead of a dominant ~23-kDa band, an ~37-kDa protein was expressed in *B. miyamotoi* HT31. Mass spectrometric analysis of the ~37-kDa protein band revealed the presence of two Vlp proteins corresponding to the Vlp15/16 protein and Vlp18 protein (WP_025444482.1 and WP_025444235.1, respectively) (Fig. 5B). This finding was confirmed by Western blot, which showed absence of anti-Vsp1 IgG binding to *B. miyamotoi* HT31 lysate (Fig. 5B). Additional Western blots using *B. miyamotoi* LB-2001–infected or –immunized mouse sera further confirmed the absence of expression of a Vsp in this isolate (Fig. 5C). Interestingly, in pooled sera from four mice infected with *B. miyamotoi* LB-2001 for 14 d, minor reactivity was observed with the *B. miyamotoi* HT31 band containing Vlp15/16 and Vlp18. However, mice immunized with LB-2001 lysate expressing Vsp1 did not produce Abs reactive with the suspected *B. miyamotoi* HT31 Vlp (Fig. 5C). This suggests that during infection with *B. miyamotoi* LB-2001, a Vlp had been expressed at some point during the infection. Additionally, 100 mg/ml anti-Vsp1 IgG did not induce killing of *B. miyamotoi* HT31 nor did it have any effect on *B. burgdorferi* viability, which further demonstrates that Vsp1 Abs specifically kill Vsp1-expressing spirochetes (Fig. 5D). However, anti-Vsp1 Ab challenge of Vsp1-expressing *B. miyamotoi* LB-2001 spirochetes consistently resulted in at least 99% of counted spirochetes to be immotile. These data collectively suggest that spirochetes that express Vsp1 are affected by anti-Vsp1 IgG Abs.
Figure 5. Vsp1 Abs neutralize Vsp1-expressing *B. miyamotoi* spirochetes, and select for spirochetes expressing a Vlp.

(A) *B. miyamotoi* LB-2001 spirochetes were incubated with different concentrations of anti-Vsp1 IgG and 25% NHS or 25% heat-inactivated serum (HIS). Five wells per condition were incubated at 37˚C, and 100 spirochetes per well were assessed by dark-field microscopy. A representative of three independent experiments is shown. (B) Coomassie blue staining of *B. miyamotoi* LB-2001 (expressing Vsp1) and of isolate HT31, expressing a ∼37-kDa protein band, which by mass spectrometric analysis was revealed to contain Vlp15/16 and Vlp18 (left panel). Western blot analysis of Vsp1 expression in LB-2001 and HT31 lysates (right panel). (C) Western blot analysis of LB-2001 and HT31 lysates using pools of sera from four mice infected for 14 d with *B. miyamotoi* LB-2001 (left), mice actively immunized with *B. miyamotoi* LB-2001 lysate (middle), or naive mice (right). A band containing Vlp15/16 and Vlp18 in *B. miyamotoi* HT31 lysate is indicated by an arrow. (D) Killing assay performed using 100 μg/ml anti-Vsp1 IgG (a-Vsp1) or a control Ab (α-OVA) and 25% NHS, on *B. burgdorferi* 297, *B. miyamotoi* HT31, and *B. miyamotoi* LB-2001. The graph is a representative of three independent experiments. (E) Coomassie blue staining of *B. miyamotoi* lysate 1, derived from *B. miyamotoi* LB-2001 previously challenged with anti-OVA, and lysate 2 derived from spirochetes surviving a challenge with anti-Vsp1 (left panel). M, protein marker. Vsp1 expression in lysate 1 and 2 as determined by Western blot using Vsp1 antiserum is shown in the right panel. (F) PCR on DNA extracted from isolates 1 and 2 (anti-OVA and anti-Vsp1 challenged, respectively) performed using an expression site promoter-specific forward primer and reverse primers for Vsp1 or VlpC2. M, marker indicating number of base pairs, with 100-bp increments. (G) Second challenge with 100 μg/ml anti-Vsp1 (α-Vsp1) or anti-OVA (α-OVA) plus 25% NHS performed on representative spirochete cultures 1 and 2 (which dominantly express Vsp1 and VlpC2, respectively). Error bars illustrate mean ± SEM. *p ≤ 0.05, **p ≤ 0.01. #p = 0.002 (X² test).
**Challenge of infected mice with anti-Vsp1 antibodies selects for a different VMP.**

We next determined whether anti-Vsp1–induced challenge would lead to a different VMP being expressed in the surviving spirochetes, similar to the relapse mechanism observed in other TBRF species. To investigate this, we incubated *B. miyamotoi* LB-2001 suspensions for 1 h with either anti-Vsp1 or a control Ab (anti-OVA, directed against OVA) and then inoculated the suspensions into MKP-F medium to culture any surviving spirochetes. Interestingly, viable spirochetes were cultured from all five replicate wells after 1 wk, however with lower concentrations in the samples previously exposed to anti-Vsp1 IgG compared with those incubated with control Ab (concentration mean ± SE, 2.4 ± 0.4 × 10^5 /ml versus 2.5 ± 0.4 x 10^6 /ml; p = 0.0006). New lysates were made from a P3 passage of these residual spirochetes, and two representative lysates are depicted, revealing survival of spirochetes expressing an ∼35-kDa band rather than Vsp1 (Fig. 5E, left panel). A Western blot confirmed that Vsp1 was only expressed in lysate 1, and not in the anti-Vsp1–exposed lysate 2 (Fig. 5E, right panel). Three peptides identified in this band by mass spectrometry matched with protein Vlp5 in strain FR64b of *B. miyamotoi* from Japan (WP_025444408.1). To obtain the *B. miyamotoi* LB-2001 homologous sequence, we performed a PCR on the isolate that was previously treated with anti-Vsp1 Abs, using a forward primer based on the “-35” promoter element and a reverse primer based on the 3’ end of the gene coding for Vlp5. This approach yielded a sequence coding for 342 aa of the dominant VMP in the expression site of the anti-Vsp1–subjected isolate. The partial gene was annotated in GenBank (KU199715; http://www.ncbi.nlm.nih.gov/genbank/), and it shared 99% nucleotide identity with the silent *B. miyamotoi* VlpC2 gene on plasmid lpE (GenBank KU041636). All four peptides derived from the mass spectrometry that were unmatched to other proteins matched with this sequence (Supplemental Fig. 1B). Subsequently, we performed PCR on the isolates subjected to control Ab (isolate 1, expressing Vsp1) or anti Vsp1 Abs (isolate 2, expressing VlpC2), amplifying only the *vsp1* or *vlpc2* genes located in the expression site. As expected, Vsp1 was amplified from the expression site in the population expressing Vsp1, whereas from the relapsed population the *vsp1* gene was not amplified downstream of the promoter (Fig. 5F). *vlpc2* was present downstream of the expression site promoter in the relapsed isolate. Interestingly, from the Vsp1-expressing population we also amplified the *vlpc2* gene in the expression site, albeit to a lesser extent. Isolates 1 and 2, expressing Vsp1 and VlpC2, respectively, were (re)exposed to anti-Vsp1 IgG or control Ab plus 25% NHS for 1 h. As expected, the *B. miyamotoi* LB-2001 isolate previously subjected to control Ab (thus still expressing Vsp1) showed a reduction in viability when exposed to anti-Vsp1. However, the *B. miyamotoi* LB-2001 isolate
that expressed VlpC2 showed no reduction in viability upon rechallenge with anti-Vsp1 IgG Abs (Fig. 5G). Taken together, these findings provide immunologic evidence that upon a borreliacidal humoral immune response to *B. miyamotoi* LB-2001 Vsp1, spirochetes that express Vsp1 are eliminated whereas surviving spirochetes that express a Vlp are unaffected, enabling them to expand. Thus, Ab-mediated selection induced a serotype switch from the dominant spirochete population to a minority serotype.

**Ab responses against Vmps in HTBRF patients**

Although all mice subjected to a Vsp1-expressing *B. miyamotoi* isolate developed high Vsp1 Ab levels, we suspected that it might not be a sensitive seromarker in humans by itself because humans might be infected by a wide range of *B. miyamotoi* serotypes, some of which could be expressing Vlps, or Vsps with low or absent cross-reactivity to Vsp1. Therefore, we assessed selected Vsp and Vlp reactivity of sera from Russian HTBRF patients in whom infection had been confirmed by sequence confirmation of *B. miyamotoi* PCR-positive blood samples. The patient population from which we were able to obtain a selection of serum samples was defined by an established case definition for *B. miyamotoi* infection (3) and has been previously described (37). In short, the first HTBRF patient sera that were PCR positive for *B. miyamotoi* during admission were collected, as well as early convalescent sera approximately 1 wk thereafter. Additionally, we also evaluated sera from Russian patients with EM (diagnosed by a physician) and healthy blood donors from the same region as controls.

Of nine HTBRF patients, all were PCR positive at the day of admission except patient Bm2, who was positive 2 d after admission. The average time between a reported tick bite and onset of disease was 16.8 d (range, 9–29 d) whereas the average time between disease onset and admission was 1.6 d (range, 1–7 d). Anti-GlpQ IgM seroconversion was observed in five of nine HTBRF patients around a week after *B. miyamotoi* was first detected by PCR in blood (Fig. 6A). Two of nine patients (Bm4 and Bm9) demonstrated a robust Vsp1-specific IgM seroconversion, one of which (Bm9) tested negative using GlpQ Abs. Another four HTBRF patients (Bm1, Bm2, Bm7, and Bm8) were demonstrated to have robust IgM seroconversion for Vlp15/16, all of whom were also positive for GlpQ, while a Vlp18 seroconversion occurred in Bm1 (Fig. 6A). Thus six of nine HTBRF patients showed a seroconversion to these *B. miyamotoi* VMPs, whereas five patients showed a seroconversion to GlpQ. Of EM patients, Bb3 demonstrated a seroconversion for GlpQ and was positive for Vlp18 IgM at admission (Fig. 6B). In addition to an EM up to 20 cm in diameter, this patient
described chills and a headache 13 d after a tick bite followed by a fever (39°C axillary temperature) and displayed elevated alanine aminotransferase (124 IU/l) and aspartate aminotransferase (94 IU/l) on admission, suggestive of a PCR-negative \textit{B. miyamotoi} coinfection. None of 12 Russian blood donors were reactive to GlpQ, Vsp1, Vlp15/16, or Vlp18 on Western blot (data not shown).

\begin{figure}
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\includegraphics[width=\textwidth]{figure6}
\caption{IgM seroconversions to Vsp1 and Vlp15/16 in acute HTBRF patients. (A) Western blots on recombinant proteins GlpQ, Vsp1, Vlp15/16, and Vlp18. Strips were incubated in sera from nine Russian patients (Bm1–Bm9) collected at the day they were first found \textit{B. miyamotoi} PCR positive in blood, and ∼1 wk thereafter. IgM was detected and all blots were developed simultaneously. The following blots were considered positive: GlpQ seroconversion, Bm1, Bm2, Bm4, Bm7, and Bm8; Vsp1 seroconversion, Bm4 and Bm9; Vlp15/16 seroconversion, Bm1, Bm2, Bm7, and Bm8; and Vlp18 seroconversion, Bm1. (B) Sera from Russian patients admitted for suspected tick-borne disease and diagnosed by a physician with EM (Bb1–Bb6) were tested for IgM against GlpQ, Vsp1, Vlp15/16, and Vlp18. The following blots were considered positive: GlpQ seroconversion in patient Bb3, GlpQ in acute and convalescent serum of Bb2, and Vlp18 in acute and convalescent serum of patient Bb3.}
\end{figure}
DISCUSSION

We report in the present study the identification of Vsp1 as an immunodominant Ag of *B. miyamotoi* LB-2001 in mice. The Ab response was efficient in clearing *B. miyamotoi* from the bloodstream in most C3H/HeN mice. Passive transfer of sera from LB-2001–infected C3H/HeN mice to *B. miyamotoi*–infected SCID mice, which normally exhibit sustained spirochetemia, induced a transient clearance of infection followed by a relapse. In vitro we showed that killing of *B. miyamotoi* LB-2001 was anti-Vsp1 mediated and largely complement-dependent. Furthermore, we demonstrated that anti-Vsp1 challenge selects for a minority of spirochetes that express VlpC2 instead of Vsp1, which become resistant to anti-Vsp1–mediated killing. Although *B. miyamotoi* and *B. burgdorferi* s.l. are present in the same tick species, the resemblance of *B. miyamotoi* to other relapsing fever spirochetes is reflected by its presence in the blood and the ability to express different immunogenic Vmps to evade humoral immunity. Taken together, our data not only provide an experimental model to study *B. miyamotoi* infection in mice, but also reveal an important role for Abs in clearance of *B. miyamotoi*. Finally, we show that Vmps induce robust Ab responses in a subset of HTBRF patients. We postulate that by combining different Vmps a serologic test could be developed to diagnose early infection in humans caused by various *B. miyamotoi* serotypes.

Spirochetemia patterns and concentrations found in our study were comparable to a study in DBA/2, C57Bl/6, and CB17 SCID mice receiving *B. miyamotoi*–infected donor mouse blood through i.v. injection, with a low-level spirochetemia after 2 d followed by undetectable spirochetemia, and a persistent spirochetemia in SCID mice (45). Although *B. hermsii* is similarly known to cause a continuous spirochetemia in SCID mice, it usually causes a more consistent pattern of relapse in wild-type mice than we observed for *B. miyamotoi* (31, 46–49). In that respect, *B. miyamotoi* is reminiscent of a type of murine relapsing fever that exhibits a similarly short-lived spirochetemia with a limited capacity for relapse (20). It is possible that other (clinical) *B. miyamotoi* isolates or serotypes will behave differently in future studies. Alternatively, *B. miyamotoi* might be less virulent than *B. hermsii* or less capable of relapsing efficiently. In line with that hypothesis, in humans a clear relapsing fever pattern has only been described in a small number of HTBRF patients. Alternatively, early antibiotic therapy in HTBRF patients might also account for the lack of relapsing disease in humans (3, 7, 37). In two severely immunocompromised and B cell–depleted patients, *B. miyamotoi* was able to cause a more chronic infection of the CNS, which has thus far not been demonstrated in immunocompetent patients (8, 9).
Our results of the passive transfer of previously infected wildtype mouse sera to infected SCID mice are comparable to those found for *B. hermsii*. Similar to what we have observed, a longer time to relapse was previously observed after transfer of late immune sera compared with transferring early immune sera (47). We speculate that this phenomenon is caused by the gradual emergence of Abs against multiple Vmps that occurs in mice during infection. In *B. hermsii*, ∼60 different silent vsp/vlp alleles are described. A switch between alleles causes a different Vmp to be expressed after spirochetemic relapse, thus evading a neutralizing IgM response against the previously expressed Vmp (20–22). Further investigation is needed to identify all Vmps in *B. miyamotoi*, and infection with clonal isolates should provide the opportunity to demonstrate active recombination. In humans, we found Abs against *B. miyamotoi* Vsp1, Vlp15/16, or Vlp18 in most of the HTBRF patients who we were able to investigate, although the contribution of cross-reactivity between yet unknown Vmp Abs remains to be investigated. Further research should elucidate how often these Vmps are recognized in larger HTBRF patient cohorts, which other Vmps are commonly expressed during human infection, the extent of Vmp cross-reactivity in Lyme borreliosis patients, and whether a combination of Vmps could increase sensitivity of HTBRF serological testing. We identified one patient who presented with an EM as well as symptoms and serology consistent with HTBRF. Although data on the incidence of coinfections are limited, studies in the United States have shown that 14% of HTBRF patients were coinfected with *B. burgdorferi*, whereas 9.8% of acute Lyme borreliosis patients were positive for GlpQ Abs (7, 14). Thus, despite a negative PCR for *B. miyamotoi*, this patient could indeed have been coinfected with *B. burgdorferi* s.l. and *B. miyamotoi*. GlpQ Abs were present in all mice and most patients in this study; however, Ab responses against Vmps were consistently more robust using our methods. Because Vsp1 Ab levels were higher than for GlpQ in mice, and were produced rapidly after infection, a Vmp-based serological assay covering the most common Vmp serotypes could improve serologic diagnosis of *B. miyamotoi* infections.

Previous studies in *B. miyamotoi* have shown both isolates LB-2001 and HT31 to be resistant to direct complement-mediated killing in vitro, similar to *B. hermsii* (12, 50). In this study, using polyclonal rabbit IgG against Vsp1, we were able to demonstrate that Ab-mediated killing was mostly complement-dependent at moderate Ab concentrations. Although complement was previously demonstrated to significantly enhance Ab-mediated killing of *B. hermsii* in vitro (49), several studies have demonstrated that complement is not essential for Ab-mediated killing and clearance of relapsing fever spirochetes, and complement- and phagocyte
independent bactericidal Abs have also been described for *B. burgdorferi*, causing outer membrane damage via a yet to be unraveled process (20, 51–55). Further studies using monoclonal IgM against Vmps should expand our understanding of this phenomenon in *B. miyamotoi*. IgM appeared to play a major role in *B. miyamotoi* clearance in wild-type mice, as its rise coincided with *B. miyamotoi* clearance, and passive transfer of 5-d immune sera to SCID mice cleared spirochetemia. In other relapsing fever spirochetes, T cell–independent IgM has a major role in early clearance, facilitated by B1b cells and splenic marginal zone B cells (21, 46, 48, 56). IgM specific for Vlp7 has been demonstrated to directly neutralize Vlp7-expressing *B. hermsii* in vitro and in vivo upon passive immunization. However, active vaccination of mice with rVlp7 did not result in protection (21). In our experiments, the humoral immune response elicited by active immunization of mice with native Vsp1 (i.e., Vsp1-expressing *B. miyamotoi* lysate) was able to temporarily clear infection upon passive transfer to infected SCID mice. Although we did not actively immunize mice with rVsp1 to assess protection, we did observe direct killing in vitro by Abs derived from rabbits actively immunized with rVsp1, and further studies should aim to identify the potential of recombinant *B. miyamotoi* Vmps in inducing protective immunity.

In conclusion, although we are only just beginning to understand the biology of *B. miyamotoi* and the (immuno)pathogenesis of HTBRF, in the present study we show that *B. miyamotoi* is able to express various Vmps to evade host humoral immune responses. The findings presented in this study provide the basis for future research on additional Vmps in *B. miyamotoi* and the scientific impetus to explore the clinical utility of a combined Vmps-based serological test for early detection of HTBRF.

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**DISCLOSURES**

The authors have no financial conflicts of interest.
REFERENCES


Supplemental figure 1

A

Supplemental figure 1. Mass-spectrometric peptide hit coverage
Mass spectrometry peptides covering (A) B. miyamotoi LB-2001 Vsp1 and (B) the partial VlpC2 amino acid sequences. Amino acid sequences that match peptides are bold and underscored.