Published in final edited form as:

Cell Microbiol. 2018 September ; 20(9): e12855. doi:10.1111/cmi.12855.

***Borrelia burgdorferi* surface-located Lmp1 protein processed into region-specific polypeptides that are critical for microbial persistence**

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Summary

One of the *Borrelia burgdorferi* virulence determinants, annotated as Lmp1, is a surface-exposed, conserved and potential multi-domain protein involved in various functions in spirochete infectivity. Lmp1 contributes to host-pathogen interactions and evasion of host adaptive immunity by spirochetes. Here we show that in diverse *B. burgdorferi* species, Lmp1 exists as distinct, region-specific and lower molecular mass polypeptides encompassing one or more domains, including independent N-terminal and middle regions and a combined middle and C-terminal region. These polypeptides originate from complex posttranslational maturation events, partly supported by a periplasmic serine protease termed as BbHtrA. While spirochete persistence in mice is independently supported by domain-specific Lmp1 polypeptides, transmission of *B. burgdorferi* from ticks to mammals requires essential contributions from both N-terminal and middle regions. Interference with the functions of Lmp1 domains or their complex posttranslational maturation events may aid in development of novel therapeutic strategies to combat infection and transmission of pathogens.

Keywords

Lyme disease; *Borrelia burgdorferi*; Lmp1; protein processing; protease pathogen; host persistence; transmission

Introduction

Lyme disease is a multi-system infection caused by the spirochete *Borrelia burgdorferi*. The disease is transmitted by *Ixodes scapularis* or related ticks, resulting in multi-system

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The authors declare that they have no competing financial interests.

complications, including arthritis, carditis and neurological disorders (Radolf *et al.*, 2012). Globally, the infection is severely underestimated and new studies suggest that, in the United States alone, there are over 300,000 new estimated cases every year (Mead, 2015). Although antibiotic treatment is usually effective, a significant fraction of patients develop persistent or relapsing symptoms, the etiology, pathogenesis and treatment options of which remains poorly understood and are controversial (Klempner *et al.*, 2013). As diagnosis of early Lyme disease remains challenging (Marques, 2015) and a human vaccine is currently unavailable (Marconi *et al.*, 2010), development of novel strategies to combat the infection is a warranted research goal.

B. burgdorferi is a unique extracellular pathogen, which exhibits a remarkable evolutionary divergence from other microbes (Paster *et al.*, 1991). Not only do spirochetes display a distinct cellular and genome organization (Fraser *et al.*, 1997, Casjens *et al.*, 2000, Casjens *et al.*, 2011, Schutzer *et al.*, 2011), but they also encode for proteins mostly of undefined function that are differentially regulated (Samuels, 2011) and support *B. burgdorferi* survival in an intricate enzootic cycle (Rosa *et al.*, 2005, Kung *et al.*, 2013). While many borrelial genes are nonessential for infectivity or transmission, some are critical (Radolf *et al.*, 2012). A surface-exposed outer membrane antigen, annotated as Lmp1, has been shown to play important roles in *B. burgdorferi* infectivity in mammals (Antonara *et al.*, 2007, Yang *et al.*, 2009, Yang *et al.*, 2010, Yang *et al.*, 2016). This chromosomally encoded outer membrane (OM) protein is a high molecular weight (~128 kDa) antigen that is conserved amongst diverse infectious strains of Lyme disease pathogens. Lmp1 features three distinct regions representing the N-terminal, middle, and C-terminal regions biological significances of all domains remain unclear. While N-terminal region supports spirochete resistance to host bactericidal effects (Yang *et al.*, 2010), the middle region facilitates microbial adhesion (Antonara *et al.*, 2007, Yang *et al.*, 2016). Notwithstanding these studies, how the full-length or individual Lmp1 regions support *B. burgdorferi* infectivity in mammals, or given the gene is expressed in the vector, its roles in tick-to-mouse transmission, remain obscure.

Our knowledge of posttranslational processing of microbial proteins has substantially advanced over the years (Cain *et al.*, 2014). However, how such mechanisms contribute in spirochete biology and infectively remain enigmatic. Recently, an ortholog of high temperature-requirement protein with serine protease activity (Clausen *et al.*, 2011) was characterized in *B. burgdorferi* (Kariu *et al.*, 2013). The protein, termed as BbHtrA or HtrABb, has been shown to play important roles in both transcriptional and posttranslational processing of multiple borrelial proteins (Coleman *et al.*, 2013, Gherardini, 2013, Kariu *et al.*, 2013b, Coleman *et al.*, 2015). BbHtrA can also be secreted extracellularly and likely cleaves specific host extracellular matrix proteins, thereby promoting spirochete dissemination (Russell *et al.*, 2013). Here we show that Lmp1 exists as region-specific polypeptides, is a substrate for BbHtrA and that Lmp1 polypeptides have independent and synergistic roles in spirochete infection and tick-to-mouse transmission. Identification of enzymes and pathways of protein maturation that are critical for infection and pathogen transmission is important for the development of new strategies to prevent Lyme borreliosis.

Results

Lmp1 exists as distinct lower molecular weight region-specific polypeptides in diverse *B. burgdorferi* strains

Lmp1 is a large protein of 1119 amino acids, which has three distinct regions, N-terminal (Lmp1N), middle (Lmp1M) and C-terminal (Lmp1C) (Fig. 1A). During detection of recombinant or native Lmp1 using immunoblot analysis, we noticed that in transfected insect cell, Lmp1 migrates as a single full-length protein of expected size (~128kDa) (Fig. 1A and Fig. S1). However, unlike in insect cells, additional lower molecular weight (MW) Lmp1 proteins, around ~100 kDa and ~47 kDa were consistently detected in immunoblot assay using spirochetes (Fig. 1B). Although these polypeptides appear to be the products of imperfect cleavage, a similar set of Lmp1 derivatives were consistently recorded in additional isolates and diverse species of infectious *B. burgdorferi* sensu lato (Fig. 1C). As we have antibodies against truncated Lmp1 proteins that detect the Lmp1N, Lmp1M, or Lmp1C regions without noticeable cross-reactivity (Yang *et al.*, 2010), these were further used to test specificity of Lmp1 processing in spirochetes. As expected, each Lmp1 region specific antibody detected full-length Lmp1 (~128kDa), as well as discrete Lmp1 derivatives. While these region-specific antibodies detected multiple fuzzy bands that are that not consistent between immunoblots, Lmp1N antibodies predominantly and constantly detected three polypeptides of ~47 kDa and Lmp1C antibodies recognized a single band with MW of ~100 kDa, Lmp1M antibodies detected both groups of truncated proteins (Fig. 1D). These results were further supported by subsequent antibody pre-absorption assays where full-length Lmp1 antibody or region-specific Lmp1 antibodies (Fig. 2A) were pre-incubated with respective recombinant proteins (Lmp1N, Lmp1M, or Lmp1C). Borrelial lysates were immunoblotted using Lmp1 antibodies pre-absorbed with specific recombinant protein, which resulted an obvious reduction of the immunoreactivity of the corresponding Lmp1 proteins (Fig. 2A), further highlighting the presence of region-specific Lmp1 truncated proteins in spirochetes. Please note that, as highlighted in Fig. 1A, Lmp1M region houses seven tandem peptide-repeats of 54 amino acids, which may be processed variably, therefore providing multiple and sometime confusing molecular weight of Lmp1 truncated proteins carrying defined number of Lmp1 peptide-repeats (Fig 2A, solid arrowheads). These truncated proteins unlikely represent protein degradation during cell lysis procedure, as similar results were consistently recorded when the spirochetes were lysed in the presence of broad-spectrum protease inhibitors.

To identify existence of truncated Lmp1 proteins, we next performed immunoprecipitation assays and eluted products were further resolved in an SDS-PAGE gel. Proteins that migrated at ~47 kDa region in the gel were excised and further subjected to LCMS/MS to identify Lmp1 polypeptides (Fig. 2B). Results show that both Lmp1N and Lmp1M peptides are detectable (Fig. 2C), but not Lmp1C, possibly due to its rapid protein turnover or weak immunogenicity. Moreover, when immunoprecipitated products were immunoblotted with antibodies raised against full-length Lmp1, Lmp1N, Lmp1M, and Lmp1C (Fig. 2D), a similar profile of truncated Lmp1 proteins as previously detected in spirochete cells (Fig. 1D) is also evidenced. The strong bands around 62kDa might be IgG heavy chain derived from immunoprecipitation procedure using protein G beads. Together, these results provided

evidence that the full-length ~128kDa Lmp1 is likely processed into two major derivatives: 1) a major ~100 kDa protein encompassing middle and C-terminal Lmp1, and 2) multiple polypeptides of ~47 kDa representing discrete N-terminal or middle regions.

Lmp1 interacts with a periplasmic serine protease BbHtrA

As the above series of data suggested that Lmp1 is subjected to proteolysis, we performed co-immunoprecipitation assays to identify potential Lmp1 interacting proteins. Spirochete lysates were immunoprecipitated with Lmp1 antibodies and eluted products were analyzed by SDS-PAGE followed by immunoblotting (Fig. 3A). A specific band (Fig. S2) that is consistently present in Lmp1 immunoprecipitated products but absent in control samples was processed for protein identification via LC-MS/MS analysis. A protease, annotated as a periplasmic chaperone-protease, BbHtrA, was identified. To confirm this, additional immunoblotting studies using anti-BbHtrA showed that BbHtrA was present in the immunoprecipitated product by anti-Lmp1 but not in controls (Fig 3B). We showed that both BbHtrA and Lmp1 localized in the periplasm of spirochetes, and that a control *B. burgdorferi* non-periplasmic protein, Rrp1, was not detectable in the fractioned periplasm (Fig. 3C). Next, assessment of Lmp1-BbHtrA interaction was also verified by independent assays. In a microtiter assay, Lmp1 region-specific proteins were bound to BbHtrA in a dose-dependent manner, which was significantly higher than control GST ($P<0.05$) (Fig. 3D). Far-western experiment further confirmed that all three Lmp1 regions interacted with BbHtrA but not a control spirochete protein BBA57, and the interaction of BbHtrA with a previously recognized substrate BB0323 (Kariu *et al.*, 2013) was recordable in our assay (Fig. 3E).

Processing of region-specific Lmp1 polypeptides involve BbHtrA

We next explored whether BbHtrA has specific cleavage activities against Lmp1. To explore this we performed a digestion assay where recombinant full-length Lmp1 was incubated with PBS, recombinant active BbHtrA, or a catalytically inactive point mutant of BbHtrA with a critical replacement of a serine residue with an alanine at the putative catalytic site (BB0104.S226A) (Fig. 4A). The results show that active BbHtrA, not the mutated version, can cleave Lmp1 into specific polypeptides, similar to previously-identified region-specific polypeptides (Fig. 1D). In contrast, BbHtrA did not cleave another control outer membrane protein of *B. burgdorferi*, BBA52 (Fig. 4A).

To confirm that BbHtrA is involved in the processing of Lmp1, we utilized isogenic spirochete mutants lacking the protease. As BbHtrA is a high, mammal-specific temperature induced protease, we cultured the wild type *B. burgdorferi*, two independent mutant clones of BbHtrA (M1, M2) and a BbHtrA-complement clone (Ye *et al.*, 2016), at 34 °C and 37 °C. Notably, at 34°C, the levels of Lmp1 proteins or derivatives reflect no obvious difference among these four isolates (Fig. 4B). However, at 37°C, wild type and BbHtrA complement isolates have reduced amount of full-length Lmp1 protein (relative density 0.16 ± 0.15) compared to BbHtrA mutants (relative density 0.6 ± 0.1 , $P<0.05$, Fig. 4C), suggesting the involvement of BbHtrA in Lmp1 processing at higher temperature. Interestingly, the wild-type spirochetes or complemented isolates showed additional ~47 kDa Lmp1 polypeptides detected using anti-Lmp1 full length, which are absent in both BbHtrA mutants (Fig. 4D).

We noticed that incubation at 37°C showed more cleaved peptide products in wild type *B. burgdorferi* compared to ones grown at 34°C. Further immunoblotting assays using Lmp1-region specific antibodies showed that ~47 kDa polypeptides were primarily represented by Lmp1N, with a minor fraction of Lmp1M, with a noticeable absence of Lmp1C (Fig. 4D). These data also corroborate the occurrence of distinct Lmp1 polypeptides in borrelial cells, as revealed by immunoblotting using Lmp1-region specific antibodies (Fig. 1D).

Lmp1 and its distinct regions contribute to the persistence of *B. burgdorferi* in mice

Although both Lmp1N and Lmp1M regions are shown to independently support spirochete infection (Yang *et al.*, 2010, Yang *et al.*, 2016), their roles in *B. burgdorferi* transmission from ticks to murine hosts remained unknown. Additionally, the role of Lmp1C, if any, during the mammalian or vector-phase of infection cycle is also unknown. Since we already generated mutants with deletion of full-length Lmp1 (Yang *et al.*, 2009) or ones producing a specific region, Lmp1N (Yang *et al.*, 2010) or Lmp1M (Yang *et al.*, 2016), herein we generate a spirochete mutant that express both N-terminal and middle regions of Lmp1, but lacking the C-terminal region by replacing part of the *lmp1* open reading frame in C-terminal with a kanamycin resistance cassette via homologous recombination (Figure 5A). The plasmid pXLF10601-*lmp1C* was generated and sequenced to confirm identity, which was transformed into wild type *B. burgdorferi*. Transformants were screened using PCR analysis to ensure the intended insertion of *kanamycin* cassette into the chromosomal locus and the presence of *lmp1 N* and *lmp1M* (Figure 5B). RT-PCR analysis showed that while *lmp1C* mRNA was absent, the mutant was able to transcribe the surrounding genes, *bb0209* and *bb0211* (Figure 5C). The *lmp1C* deletion mutant spirochetes contained a similar protein profile to that of the wild type (Figure 5D, left panel), and the *lmp1C* mutant did not produce Lmp1 full length protein (Figure 5D, right panel). To confirm the expression of Lmp1 N-terminal and middle region in *lmp1C* deleted mutants, western blotting using anti-Lmp1 full length antibodies were performed. The results showed that *lmp1C* deletion mutants produced three ~47kD fragments and also a larger protein above 62kD that might be the Lmp1N+M fusion protein (Figure 5E). The *lmp1C* mutant clone with identical plasmid profiles as wild type was used for further study (data not shown). The genetic manipulation did not affect the growth of *lmp1C* mutants (data not shown). Next, to assess relative fitness of region-specific Lmp1 mutants, five groups of mice (3 animals/group) were infected with equal numbers (10^5 cells/ mouse) of the wild type, Lmp1 deletion mutant, Lmp1N complement, Lmp1M complement or Lmp1C deletion (Lmp1N+M) bacteria. Spirochete levels in murine skin, heart, joint, and bladder tissues were measured at day 21 following infection. Murine joint swellings were also monitored throughout the infection. As showed in Fig. 6A, although the Lmp1 mutants remained infectious, they had reduced spirochetal burden in tissues compared to wild-type *B. burgdorferi* ($P<0.05$). Interestingly, the full-length Lmp1 deletion showed the most obvious defect in spirochete persistence in murine tissues compared to isolates carrying truncated Lmp1 proteins complemented with any of the Lmp1 domain (Fig 6A). These studies suggest while each Lmp1 domain independently supports *B. burgdorferi* infectivity, function of full-length protein is required for optimal microbial persistence in the host. Compared to the mice infected with wild type, none of the Lmp1 mutants were able to induce significant ankle swelling in mice ($P<0.05$, Fig. 6B).

Both N-terminal and middle regions of Lmp1 is necessary for spirochete transmission from ticks to mice

As *lmp1* is highly expressed in ticks (Yang *et al.*, 2009), and recent publication indicate that deletion of *Lmp1* significantly affects the persistence of spirochete in ticks (Koci *et al.*, 2018). We sought to assess the role of *Lmp1* regions in *B. burgdorferi* transmission through ticks to murine hosts. As mutants are impaired for persistence in mice and thus cannot be acquired in ticks at similar levels to the wild type, we microinjected equal concentrations of wild type or *lmp1* mutants in naïve ticks and placed on mice. The fully replete ticks were collected and at 12 days after tick feeding, spirochete infection in mice was assessed. Unlike other three groups, the wild type and *Lmp1*N+M isolate showed strong serological responses (Fig. 7A). Spirochete levels were also detected in all collected tissues only for wild type and *Lmp1* N+M isolates but not for other *lmp1* mutants (Fig. 7B). These results confirmed that contribution of both *Lmp1*N and *Lmp1*M regions are essential for *B. burgdorferi* transmission through ticks to host.

Discussion

Lmp1 is a known borrelial virulence determinant with multiple proposed functions ranging from evasion of host adaptive immunity to host-pathogen interactions (Antonara *et al.*, 2007, Yang *et al.*, 2009, Yang *et al.*, 2010, Yang *et al.*, 2016). Available *in silico* and experimental data suggest that *Lmp1* harbors three distinct functional regions (Yang *et al.*, 2016). While *Lmp1* N-terminal region (*Lmp1*N) contributes to spirochete immune evasion (Yang *et al.*, 2010), the middle region (*Lmp1*M) binds to host chondroitin-6-sulfate molecules thereby acting as a microbial adhesin (Yang *et al.*, 2016). These results are supported by mutagenesis studies highlighting that both *Lmp1*N and *Lmp1*M can function independently to support infectivity of *B. burgdorferi* (Yang *et al.*, 2010, Yang *et al.*, 2016). Here we present evidence that *Lmp1* exists as separate, region-specific and lower MW polypeptides that include at least one ~100 kDa and three ~47 kDa polypeptides. Our data also suggest that these polypeptides are likely the results of post-transcriptional proteolysis events, a process that is supported, at least partly by a periplasmic serine protease BbHtrA (Coleman *et al.*, 2013, Kariu *et al.*, 2013b, Russell *et al.*, 2013). We further confirmed that while ~100 kDa peptide constitutes both regions of *Lmp1*M and *Lmp1*C, smaller ~47 kDa polypeptides primarily represent *Lmp1*N. Using newly created *lmp1* mutants, we show that each of the *Lmp1* domain contributes to spirochete infectivity. Our recent publication using an artificial tick feeding system demonstrates that *Lmp1* contributes to the persistence of *B. burgdorferi* in ticks (Koci *et al.*, 2018). Our present study further shows that *Lmp1*C region is required for optimal *Lmp1* function in mammals, but redundant in the vector where *Lmp1*N and *Lmp1*M regions are sufficient to support pathogen transmission from ticks to murine hosts. Together, these studies shed new light on the biology of *Lmp1* and its cellular processing aiding in development of novel therapeutic strategies to combat infection.

The *lmp1* (*bb0210*) gene lacks a discernible promoter and is likely transcribed as a bicistronic transcript with the upstream *bb0209* mRNA driven by an obvious promoter (Yang *et al.*, 2009). Although there is a possibility that *Lmp1* polypeptides are derived from multiple truncated *lmp1* transcripts, as previously shown for complex transcription events in

spirochetes (Dobrikova *et al.*, 2001), our evidence supports the posttranslational proteolytic processing Lmp1. First, Lmp1 immunoprecipitated products from spirochetes identified a periplasmic chaperone-protease, BbHtrA (Coleman *et al.*, 2013, Kariu *et al.*, 2013b, Russell *et al.*, 2013), as an Lmp1 interacting protein. BbHtrA accelerated cleavage of full-length Lmp1 in *in vitro* digestion assays, but not another borrelial outer membrane protein, BBA52 (Kumar *et al.*, 2010). The Lmp1 cleavage activity was absent in a catalytic BbHtrA mutant (Kariu *et al.*, 2013b). Second, processing of Lmp1 in spirochetes was impaired in isogenic BbHtrA-deficient mutants (Ye *et al.*, 2016) but was comparable in wild-type and BbHtrA-complemented isolates; notably, the difference of Lmp1 processing was not recorded in spirochetes grown at 34°C but obvious at 37°C where protease activity of BbHtrA is known to be activated (Clausen *et al.*, 2002, Clausen *et al.*, 2011, Coleman *et al.*, 2013, Kariu *et al.*, 2013b). This also suggests an artificial or nonspecific cleavage during cell lysis is unlikely as our preparations were performed at room temperature (where BbHtrA or other proteases are inefficient) and also includes the presence of protease inhibitors. Third, both Lmp1 and BbHtrA are detectable in the same borrelial subcellular compartment (periplasmic space), where processing of Lmp1 polypeptides likely to occur. Fourth, a mass spectrometry analysis of spirochete proteins excised from ~47 kDa region identified peptides predominantly from Lmp1N and also from Lmp1M, further corroborating the immunoblot data on their relative existence in borrelial cells. Fifth, a similar Lmp1 processing pattern has been recorded in all tested diverse strains of spirochetes. Finally, additional Lmp1 polypeptides close to 100 kDa likely represent the polypeptides housing Lmp1M of various length, which have seven repeats of 54 amino acids and likely features potentially imperfect or multiple cleavage sites (Yang *et al.*, 2016). Although deletion of BbHtrA impacted the occurrence of full-length Lmp1 as well as polypeptides, a ~47 kDa polypeptide is still present in BbHtrA mutants, which based on immunoblotting likely to represent the Lmp1N region. Therefore, existence of Lmp1N (or possibly other undetectable polypeptides) in BbHtrA mutants suggested that an unidentified mechanism of Lmp1 processing also occurs in a BbHtrA independent manner, for example in ticks where enzymatic activities of BbHtrA might be minimal due to colder temperature. Another possibility is that there is an unidentified internal promotor site that expresses ~47 kDa polypeptide. As HtrA proteins also acts as chaperones for stabilizing specific proteins (Clausen *et al.*, 2002, Clausen *et al.*, 2011) and the fact that we show this chaperone-protease directly binds all three regions of Lmp1 (Lmp1N, Lmp1M and Lmp1C), it is tempting to speculate that BbHtrA is also involved in the stabilization of specific Lmp1 polypeptides essential for infection.

In conclusion, our study underscores existence of complex posttranslational mechanisms for maturation and/or stability of spirochete gene-products that impact spirochete infection and transmission. Relatively less is known about posttranslational processing of virulence determinants in *B. burgdorferi* (Samuels *et al.*, 2010). In fact, Lmp1 is amongst a handful of spirochete gene-products that is known to undergo proteolytic or other modes of protein maturation events. Lmp1 is a conserved, chromosomally-encoded protein (Fraser *et al.*, 1997) required for establishment of persistence infection in mammals, or transmission from infected ticks to murine hosts, and also predicted to be a vaccine candidate against diverse *B. burgdorferi* species (Poljak *et al.*, 2012). Therefore, further understanding of the maturation process of a potential multi-functional protein like Lmp1, for its role in the host-pathogen

interaction or other unknown functions, will enrich our knowledge of intriguing biology and infectivity of spirochetes. As each of Lmp1 regions independently or in combination support spirochete infection and transmission, future studies on the identification of inhibitors that block of Lmp1 processing, including Lmp1-BbHtrA interactions, or their functions may also contribute to the development of new therapeutic strategies to combat *B. burgdorferi* infection.

Experimental Procedures

B. burgdorferi, Mice and Ticks

Low-passage *B. burgdorferi* isolates, B31-A3 and 297-AH130, N40, *B. afzelii*, and *B. garinii* were used in this study (Pal *et al.*, 2004, Yang *et al.*, 2009). Bacteria were cultivated in Barbour-Stoenner-Kelly II (BSK-II) medium at 34°C or at 37°C. For genetic manipulation and maintenance, Lmp1- or BbHtrA-deficient spirochetes were cultivated with appropriate antibiotics (Yang *et al.*, 2009, Yang *et al.*, 2010, Yang *et al.*, 2016, Ye *et al.*, 2016). Four- to six-week-old C3H/HeN mice were purchased from the National Institutes of Health and Charles River laboratories. Animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee and Institutional Biosafety Committee of the University of Maryland. *I. scapularis* ticks used in this study originated from a colony maintained in our laboratory.

Polymerase Chain Reaction

The PCR and quantitative RT-PCR (qRT-PCR) assays are performed as detailed (Yang *et al.*, 2009). Total RNA from cultured spirochetes or from infected tissues was isolated with TRIzol reagent (Life Technologies), treated with DNase I (NEB), reverse transcribed to cDNA (SuperScript VILO; Invitrogen), and analyzed by real-time PCR using SYBR green Supermix (Life Technologies) as detailed (Yang *et al.*, 2009). For quantification of spirochete levels in mouse tissues, *flaB* transcripts were used as surrogate and normalized to mouse or tick β -actin levels (Yang *et al.*, 2009).

Enzyme-linked immunosorbent assay

ELISA assays were performed as detailed (Singh *et al.*, 2016). Plates were coated with target proteins, blocked with PBS-T (0.05% Tween 20, 5% normal goat serum in PBS, pH 7.4) and exposed to diluted sera (1:5,000) in PBS-T. Signal was detected using Horseradish peroxidase (HRP)-conjugated secondary antibodies and substrates, as detailed (Singh *et al.*, 2016).

Immunoblotting and far-Western blotting assays

These assays were detailed earlier (Kariu *et al.*, 2013a, Kariu *et al.*, 2015). For immunoblotting, proteins from cell lysates or periplasm (PeriPreps™ Periplasting Kit, Epicentre) were electrophoresed and immunoblotted with diluted appropriate primary and secondary HRP-conjugated antibodies, and signals developed using chemiluminescence as described (Kariu *et al.*, 2013a, Kariu *et al.*, 2015). For far-Western analyses, before addition of primary antibodies (anti-BbHtrA), the membrane was incubated with recombinant

BbHtrA (2 µg/ml) followed by primary and secondary antibodies, as detailed (Kariu *et al.*, 2013b).

Immunoprecipitation and LC-MS/MS analysis

Immunoprecipitation assays of spirochete proteins by anti-Lmp1 antibodies were performed as detailed (Yang *et al.*, 2011) using a commercially available product (Sigma Protein G Immunoprecipitation Kit). The *B. burgdorferi* were collected and lysed by resuspension in 1× BugBuster Reagent (EMD Biosciences). The protein G beads were then incubated with pre-cleared spirochete lysates and Lmp1 antisera or normal mouse sera 4°C overnight. After immunoprecipitation, the bound proteins were eluted in 50 µL sample buffer [62 mM Tris-HCl (pH 6.8), 10% v/v glycerol, 100 mM DTT, 2% SDS, 0.001% bromophenol blue], subjected to SDS-PAGE and immunoblot assays. Excised proteins from SDS-PAGE gels were identified via LC-MS/MS as detailed (Yang *et al.*, 2011).

Generation and phenotypic analysis of *B. burgdorferi* mutants

Genetic manipulation of spirochetes involved our published procedures (Yang *et al.*, 2009, Yang *et al.*, 2010, Yang *et al.*, 2016). A *B. burgdorferi* mutant lacking C-terminal Lmp1 region was generated by homologous recombination, replacing a fragment of Lmp1C with a kanamycin resistance cassette, using primers indicated in Supplementary Table S1. The 5' and 3' arms of *lmp1* (2325–3261 base pair) were amplified and cloned into multiple cloning sites flanking the kanAn cassette in pXLF10601 (Yang *et al.*, 2009). The plasmid was electroporated into spirochetes and clones that grew in antibiotic-containing media were tested for the intended recombination event, loss of Lmp1, and presence of endogenous plasmids. Growth of spirochetes was monitored using dark-field microscopy (Yang *et al.*, 2010).

Proteolytic digestion assay

A proteolytic digestion assay was performed as detailed (Kariu *et al.*, 2013b). Briefly, 300 ng of recombinant wild-type BbHtrA or its enzymatically inactive version was incubated with various concentration of recombinant Lmp1 in digestion buffer (50 mM Tris-HCl pH 7.0, 1 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol) at 37°C. The reactions were terminated at various times and digests were analyzed by immunoblotting.

Infection studies

Groups of three C3H/HeN mice were injected intradermally with an equal number (10⁵ cells/mouse) of wild-type spirochetes or specific *lmp1* mutants (Yang *et al.*, 2010). For tick transmission studies, nymphs were microinjected with equal number of wild type, *lmp1* mutant, *lmp1* mutant carrying N-terminal domain, *lmp1* mutant carrying middle region, or *lmp1* mutant carrying both N-terminal and middle region but lacking C-terminal domain. After overnight incubation, the microinjected ticks were allowed to feed on mice (5 ticks/mouse). After tick complete replete, all ticks were collected. The mouse skin, heart, joint and bladder were collected at 14 days after tick feeding, and pathogen levels were assessed by qRT-PCR analysis of *flaB* transcripts normalized to murine β -actin levels (Kariu *et al.*, 2015).

Statistical analysis

All numerical results are expressed as the mean standard error of the mean (SEM). The significance of the difference between the mean values of the groups was evaluated by Student's t test with GraphPad Prism software, version 4.0 (GraphPad Software, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by R01AI080615 and R01AI116620 (to U. P.), R03AI103894 and R03AI092143 (to X. Y.) X.Z. is the recipient of the Deborah and Mark Blackman Postdoctoral Fellowship from Global Lyme Alliance.

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Figure 1. Lmp1 exists as distinct multiple smaller molecular weight polypeptides in *B. burgdorferi*

(A) Schematic drawing of potential mature region-specific Lmp1 proteins. The upper panel denotes the full-length protein, indicating positions of a 26-mer signal peptide, as identified by in silico analysis, N-terminal region (Lmp1N), a middle region (Lmp1M) with seven tandem peptide-repeats of 54 amino acids (gray boxes), and a C-terminal region (Lmp1C) containing seven tetratricopeptide repeat (TPR) motifs. The lower two panels show organization of possible mature Lmp1 polypeptides containing Lmp1N+Lmp1M and individual Lmp1N+Lmp1M, respectively. (B) Recognition of Lmp1 protein by full-length Lmp1 antibodies. Lysates from insect cells producing recombinant Lmp1 (left lane) and cultured *B. burgdorferi* (Bb) cells (right lane) were immunoblotted using anti-Lmp1 antibodies. The Lmp1 antibodies react with a protein band around 128kDa in transfected insect cells or in spirochetes (arrow). Note that Lmp1 antibodies also recognize additional several lower molecular weight (MW) proteins of ~100 kDa and ~47 kDa proteins (arrowheads) absent in insect cells. (C) Lmp1 proteins in diverse isolates and strains of *B. burgdorferi*. Equal levels of total proteins from various *B. burgdorferi* isolates were immunoblotted with Lmp1 antibodies. A similar set of Lmp1 proteins or lower MW polypeptides, as shown in panel 1B (arrow and arrow-heads) were detected in diverse isolates of infectious Lyme disease pathogens, except for in N40, where the full-length protein is undetectable. The short-term exposure of chemiluminescence substrate to HRP enzyme (low exposure) showed clearly three processed polypeptides (middle panel). Equal

protein loading was indicated by immunoblotting using FlaB antibodies. **(D)** Detection of fulllength and smaller region-specific Lmp1 polypeptides. Recognition of full-length Lmp1 (arrowhead) and derivatives (arrows) were achieved by antibodies generated against specific Lmp1 regions (N-terminal, Middle and C-terminal). Noted that the strong bands around 62kDa are likely to be mouse IgG. Equal amount of lysates from wild type (WT) and an isogenic *lmp1* deletion mutant (M) were probed with antibodies. Protein loading is indicated by immunoblotting using FlaB antibodies.

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Figure 2. Identification of Lmp1 protein and polypeptides by antisera

(A) Recognition of Lmp1 protein and region-specific polypeptides studied by antibody pre-absorption. Antibodies against specific Lmp1 regions (anti-Lmp1N, anti-Lmp1M, or anti-Lmp1C) (left panel) or antifull-length Lmp1 (right panel) were pre-absorbed with respective truncated recombinant Lmp1 proteins (Lmp1N, Lmp1M, or Lmp1C) (lanes indicated as +). Pre-absorbed antibodies or ones without any pre-absorption (lanes indicated –) were used for immunoblotting against *B. burgdorferi* lysates. Anti-FlaB was used for loading control. Arrow, open arrow-heads, and solid arrow-heads denotes full-length Lmp1, Lmp1N, and multiple Lmp1 truncated proteins carrying defined number of Lmp1M peptide-repeats (Fig. 1A), respectively. Lmp1M (B) Identification of lower molecular weight Lmp1 polypeptides identified by immunoprecipitation (IP). The Lmp1 antibodies were used to immunoprecipitate Lmp1 polypeptides from *B. burgdorferi* lysates and were analyzed by SDS-PAGE; products around 47kD were excised for LCMS/MS analysis. (C) Amino acid sequence of N-terminal part of Lmp1 showing the peptides identified by LC-MS/MS analysis. The sequence and location of peptides in Lmp1N (amino acids 1–380) and Lmp1M (beginning from amino acids 318, indicated by an asterisk) are shown. The peptides were underlined and the first and terminal residue indicated in bold face. (D) Presence of full-length and region-specific Lmp1 polypeptides in *B. burgdorferi*. The IP studies were performed as detailed in Fig. 2B and immunoprecipitated products were analyzed by immunoblotting using antibodies against full-length or truncated region-specific Lmp1 polypeptides.

Figure 3. Occurrence of Lmp1 in *B. burgdorferi* periplasm and interaction with BbHtrA protease (A) Immuno-precipitation of Lmp1 proteins by anti-Lmp1 antibodies. *B. burgdorferi* lysates were immunoprecipitated with Lmp1 antibodies or with control normal mouse sera (NMS), and subjected to immunoblotting using anti-Lmp1 (left panel) or antisera raised against *B. burgdorferi* lysates (right panel). The *B. burgdorferi* lysates immunoblotted with antisera against *B. burgdorferi* was used as control. The bands that specifically immunoprecipitated with anti-Lmp1 or ones that are only present in IP products using anti-*B. burgdorferi* were processed for LC-MS/MS (arrowheads). Those two major bands are likely to be IgG heavy chain and light chain derived from IP using protein G beads. (B) BbHtrA is immunoprecipitated by anti-Lmp1. BbHtrA is detectable in the immunoprecipitated products by anti-Lmp1 but not in NMS control using anti-BbHtrA. (C) BbHtrA and Lmp1 are detectable in periplasmic fraction of *B. burgdorferi*. Whole cell lysates (Cell) and periplasmic fraction (Peri) were isolated from spirochetes and immunoblotted with anti-Lmp1 (upper panel), anti-BbHtrA (middle panel) or anti-Rrp1 (lower panel) antibodies. (D) ELISA was used to detect the interaction between three Lmp1 regions and BbHtrA. The wells were coated with recombinant BbHtrA protein and incubated with increasing (6.25–1000 ng) concentrations of Lmp1N, Lmp1M, Lmp1C or GST (control) proteins. Compared

to GST control, addition of similar amount of Lmp1 truncated protein showed significant higher binding to BbHtrA (* $P < 0.05$). (E) Interaction between Lmp1 region-specific proteins and BbHtrA were examined by far-Western assay. The Lmp1N, Lmp1M, Lmp1C and a negative control protein (BBA57) and a positive control protein (BB0323N) were probed with recombinant BbHtrA and binding was detected using anti-BbHtrA sera.

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Figure 4. The BbHtrA-mediated processing of Lmp1 protein

(A) Digestion of Lmp1 by recombinant BbHtrA. Recombinant BbHtrA and Lmp1 proteins were incubated and Lmp1 digests were analyzed by immunoblotting. Note that the mutated version of BbHtrA (middle panel) lacks the ability to digest full-length Lmp1, and the wild-type BbHtrA failed to digest another *B. burgdorferi* outer membrane protein, BBA52 (right panel). (B) Detection of Lmp1 and processed polypeptides in BbHtrA-deficient spirochetes grown at 34°C. Lysates from wild type (WT), two independent clones of BbHtrA mutants (M1 and M2) and a complemented isolate (Com) were grown at 34°C and immunoblotted with anti-Lmp1 antisera. Anti-FlaB was used for loading control and anti-BbHtrA was used to confirm depletion of the protein in the mutants. Almost equal levels of 100kDa (arrow) and a single 47kDa band (arrowhead) are present in all groups of spirochetes. (C) Decreased Lmp1 processing in BbHtrA-deficient spirochetes grown in 37°C. Immunoblotting of spirochetes grown at 37°C using anti-Lmp1 antisera was performed (upper panel). The densitometric analysis (lower panel) suggested a greater abundance of full-length Lmp1 (arrow) in both BbHtrA mutant clones (M1 and M2) compared to WT and BbHtrA complemented isolate (Com) (*P<0.05). (D) Immunoblotting using region-specific Lmp1 antibodies. BbHtrA-deficient spirochetes were immunoblotted with antisera against full

length and each region-specific anti-bodies. The ~47kDa polypeptides (arrowhead) are mostly represented by Lmp1N, and to some extent Lmp1M, while absent by Lmp1C.

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Figure 5. Construction and analysis of a *Lmp1* mutant without a C-terminal region

(A) Schematic representation of wild type (WT) and a mutant lacking the C-terminal region (Lmp1C). The Lmp1C was replaced in spirochetes by an antibiotic cassette (KanAn) using allelic exchange. The numbers indicate the P1-P4 primer location in *B. burgdorferi* genome (black arrowhead) for generation of mutagenic constructs. The primer P5-P9 (blank arrow head) used for verification of correct integration of KanAn are indicated. All primer sequence was listed detailed in Table S1. (B) PCR analysis of mutants to check desired integration of KanAn. The DNA samples from *lmp1C* mutant clone were subjected to PCR using various sets as indicated. (C) RT-PCR analysis. Total RNA was isolated from WT, and *lmp1C* mutant *B. burgdorferi*, transcripts of *bb0209*, *bb0211*, *lmp1* and *flaB* were analyzed using RT-PCR. (D) Protein profile and Lmp1C expression. Wild type (WT) and *lmp1C* mutant (M) *B. burgdorferi* protein were separated using SDS-PAGE gel and either stained with Coomassie blue (Left panel) or immunoblotted with anti-Lmp1C and anti-FlaB antisera (Right panel). (E) Production of truncated Lmp1 protein encompassing N-terminal and middle regions. Anti-Lmp1 serum was used to detect protein expression of WT and Lmp1C (M) deleted mutant *B. burgdorferi*. FlaB was used for loading control.

Figure 6. Lmp1 and its three distinct regions are important for *B. burgdorferi* persistence in mice
(A) Infection in mice. Groups of mice were injected with wild type (WT, black bars), the mutant with deletion of full-length Lmp1 (Lmp1⁻, light grey bars), or Lmp1N complement (Lmp1N⁺, gray bars), Lmp1M complement (Lmp1M⁺, dark gray bars) and Lmp1C deleted mutant (Lmp1NM⁺, white bars) *B. burgdorferi* strains. The spirochete burden was analyzed at day 21 in heart, joint, skin, and bladder tissues following spirochete infection. The *B. burgdorferi* burden was significantly reduced in all mouse tissues infected with *Lmp1* mutants compared to wild type (*P<0.05). Bars represent the mean $\pm$ SEM of four quantitative RT-PCR analyses of *B. burgdorferi* levels derived from two independent animal

experiments. **(B)** Assessment of joint swelling. The ankle swelling in mice infected with wild type or mutant spirochetes was monitored at various time points after infection. The joint size was significantly lower compared wild type (* $P < 0.05$). Bars represent the mean $\pm$ SEM of three independent mice from one of two independent animal experiments.

Figure 7. The amino terminal and middle region of Lmp1 are essential for spirochete transmission from ticks to mice

(A) Serological response in the infected mice. Ticks microinjected with wild-type (WT), the mutant with deletion of full-length Lmp1 (Lmp1⁻), or one complemented with Lmp1N (Lmp1N⁺) or middle domain (Lmp1M⁺) or one with deletion of Lmp1C (Lmp1NM) were fed on groups of mice. Sera were collected at day 12 after tick engorgement and immunoblotting was performed to assess the transmission of *B. burgdorferi*. (B) Assessment of *B. burgdorferi* burden. The samples of skin, heart, joint and bladder tissues were collected from mice infected with various isolates (as indicated in 7A) and assessed for *B. burgdorferi* levels using qPCR. The *B. burgdorferi* burden was significantly reduced in all mouse tissues

transmitted by ticks infected with Lmp1⁻, Lmp1N⁺ and Lmp1M⁺ compared to WT and Lmp1NM (*P<0.05). Bars represent the mean $\pm$ SEM of four qPCR analyses of *B. burgdorferi* levels derived from two independent animal experiments.

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