Tick-host-pathogen interactions in Lyme borreliosis
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Preferential protection of *Borrelia burgdorferi* sensu stricto by a Salp15 homologue in *Ixodes ricinus* saliva

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Abstract

Background. *Ixodes* ticks are the main vectors for *Borrelia burgdorferi* sensu lato. In the United States, *B. burgdorferi* is the sole causative agent of Lyme borreliosis and is transmitted by *Ixodes scapularis*. In Europe, 3 *Borrelia* species – *B. burgdorferi*, *B. garinii*, and *B. afzelii* – are prevalent, which are transmitted by *Ixodes ricinus*. The *I. scapularis* salivary protein Salp15 has been shown to bind to *B. burgdorferi* outer surface protein (Osp) C, protecting the spirochete from antibody-mediated killing. Methods and results. We recently identified a Salp15 homologue in *I. ricinus*, Salp15 Iric-1. Here, we have demonstrated, by solid-phase overlays, enzyme-linked immunosorbent assay, and surface plasmon resonance, that Salp15 Iric-1 binds to *B. burgdorferi* OspC. Importantly, this binding protected the spirochete from antibody-mediated killing in vitro and in vivo; immune mice rechallenged with *B. burgdorferi* preincubated with Salp15 Iric-1 displayed significantly higher *Borrelia* numbers and more severe carditis, compared with control mice. Furthermore, Salp15 Iric-1 was capable of binding to OspC from *B. garinii* and *B. afzelii*, but these *Borrelia* species were not protected from antibody-mediated killing. Conclusions. Salp15 Iric-1 interacts with all European *Borrelia* species but differentially protects *B. burgdorferi* from antibody-mediated killing, putatively giving this *Borrelia* species a survival advantage in nature. 
Introduction

Ticks are ectoparasites that, while taking a blood meal, can transmit a variety of human pathogens. Lyme borreliosis is a common tickborne disease in the United States and Europe, where *Ixodes scapularis* and *Ixodes ricinus* are the most important vectors, respectively. In the United States, *Borrelia burgdorferi* sensu stricto (*B. burgdorferi*) is the only prevalent *Borrelia* species and is transmitted by *I. scapularis*, whereas in Europe 3 *Borrelia* species – *B. burgdorferi*, *B. garinii*, and *B. afzelii* – can cause Lyme borreliosis and are transmitted by *I. ricinus*. In humans, all 3 species frequently cause an erythematous cutaneous lesion, erythema migrans. In later stages of infection, spirochetes can disseminate and cause disease that affects the joints, cardiac conduction system, central nervous system, and skin [1, 2]. It has been demonstrated previously that each *Borrelia* species is associated with distinct clinical entities [1, 3, 4].

During its enzootic life cycle, *B. burgdorferi* exploits tick salivary proteins [5]. These vector molecules are important for *B. burgdorferi* survival within the tick (e.g., TROSPA [6]), for transmission from the host to the tick (e.g., Salp25D [7]), and for transmission from the tick to the host (e.g., Salp15 [8]). Salp15 is a 15-kDa *I. scapularis* feeding-induced salivary protein [9] and has been shown to bind to *B. burgdorferi* outer surface protein (Osp) C [8]. *B. burgdorferi* expresses OspC in the tick salivary glands and during the early stages of mammalian infection [10]. Binding of Salp15 to OspC protects the spirochete from antibody-mediated killing by the host. In nature, the ability of *B. burgdorferi*, assisted by *I. scapularis* Salp15, to reinfect immune reservoir hosts could be an important factor in the continuation of the complex enzootic life cycle of the spirochete.

In addition to *B. burgdorferi*, *I. ricinus* is also able to transmit 2 other *Borrelia* species that cause Lyme borreliosis, *B. garinii* and *B. afzelii*. Recently, we identified 3 Salp15 homologues in *I. ricinus*, Salp15 Iric-1, -2, and -3, of which Salp15 Iric-1 is most similar to *I. scapularis* Salp15 [11]. In the present study, we describe the interaction of Salp15 Iric-1 with its presumed natural ligand, OspC from *Borrelia burgdorferi* sensu lato strains representing the 3 pathogenic *Borrelia* species in Europe.

Methods

*I. ricinus* Salp15 homologue–specific reverse-transcription polymerase chain reaction (RT-PCR). Adult female *I. ricinus* ticks were fed on rabbits. After 3 days, semiengorged ticks were removed, salivary glands were dissected, and RNA was isolated and cDNA generated as described elsewhere [11]. Quantitative RT-PCR on *I. ricinus* salivary gland cDNA was performed using different sets of primers specific for the 3 Salp15 variants (table 1). Amplification of cDNA- and gene-specific standards was visualized and quantified using LightCycler software (Roche Diagnostics).

Cloning and expression of Salp15 Iric-11. Salp15 Iric-1 (GenBank accession number EU128526) was amplified from a recombinant pGEM-T Easy vector (Promega) (table 1), inserted into the
Chapter 5

Table 1. Primers and probe used.

<table>
<thead>
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<th>Target</th>
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<th>Reverse</th>
<th>Probe</th>
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<tbody>
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</tr>
<tr>
<td>Irac-2 specific</td>
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<td>-</td>
</tr>
<tr>
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</tr>
<tr>
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<td>AAAAAAACGCAGCGCCACA-</td>
<td>-</td>
</tr>
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<td>AAAAAATCTCGAGCTAAG-</td>
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<td>AAAAAATCTCGAGCTAAG-</td>
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<td>Borrelia flaB</td>
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<td>TCGTCTGTAAGTGGCTCTATTTT</td>
<td>GAATTRGC</td>
</tr>
<tr>
<td>Mouse β-actin</td>
<td>GGGACCTGACAGACTACCTC</td>
<td>AAGAAGGAGGGCTGGAAAAG</td>
<td>-</td>
</tr>
</tbody>
</table>

pMT/BiP/V5–His C vector (Invitrogen), transformed into DH5-α cells that were subjected to plasmid isolation (Miniprep Kit; Qiajen), and sequenced as described elsewhere [11]. Drosophila S2 cells (Invitrogen) were cotransfected with a blasticidin selection vector, pCOBlast (Invitrogen). Stably transfected cells were grown and induced and recombinant protein was purified as described elsewhere [8]. I. scapularis Salp15 (GenBank accession number AF209914) was generated in a similar fashion. Protein concentrations were determined using a standard Bradford assay, Coomassie staining, and Western blot analysis with anti-V5–horseradish peroxidase (HRP) antibody. Mock-transfected Drosophila S2 cells were grown and induced, and the supernatant was run over a purification column to generate a “vehicle control” to control for impurities (i.e., Drosophila background proteins) in the recombinant protein fractions. Where indicated, deglycosylation was performed by use of N-glycosidase F (New England Biolabs) under denaturing conditions.

Borrelia burgdorferi sensu lato. B. burgdorferi sensu lato strains representing the 3 pathogenic Borrelia species that exist in Europe - B. burgdorferi strains N40 and B31 clone 5A11 [12], B. garinii strain PBi, and B. afzelii strain pKo - were cultured in Barbour-Stoenner-Kelly (BSK)-H medium (Sigma-Aldrich). Low-passage spirochetes were grown to 5 x 10^7 organisms/mL (enumerated by use of a Petroff-Hauser counting chamber, as described elsewhere [13]) and diluted to the indicated concentrations.

Solid-phase overlay. Borrelia lysates were separated by 12.5% SDS-PAGE and blotted onto an Immobilon-P membrane (Millipore) to have approximately similar amounts of OspC. As a negative control, we used lysate from ospC-deficient B. burgdorferi strain 297 [14]. Solid-phase overlays were performed as described elsewhere [8]. In addition, Western blot analysis was performed using a monoclonal antibody, L22 1F8, recognizing OspC from B. burgdorferi sensu lato [15].

Cloning and expression of recombinant B. burgdorferi sensu lato OspC. OspC was amplified from genomic DNA from Borrelia strains N40, PBi, and pKo (table 1) and inserted into the pGEX-
Preferential protection of *Borrelia burgdorferi* by *Ixodes ricinus* Salp15

6p-2 vector (Amersham Biosciences). Recombinant plasmids were cloned into DH5-α cells, and inserts were sequenced as described elsewhere [11]. Large cultures were induced by use of isopropyl β-D-1-thiogalactopyranoside (final concentration, 1 mmol/L). Recombinant glutathione S-transferase (GST)–fused OspC proteins were harvested, purified, and cleaved from GST in accordance with the manufacturers’ recommendations. Purity was checked using SDS-PAGE, and protein concentrations were measured using a Bradford assay.

**OspC-Salp15 ELISA.** Microlon ELISA plates (Greiner) were coated with recombinant OspC from the 3 *Borrelia* strains (400 ng/well) or with bovine serum albumin (BSA) as a control in 100 μL/well coating buffer (0.1 mol/L Na₂CO₃ [pH 9.6]) overnight at 4°C. Subsequently, wells were washed (wash buffer, PBS plus 0.05% Tween) and incubated with 200 μL of blocking buffer (10% fetal bovine serum in PBS) for 2 h. Subsequently, 50 μL of blocking buffer with *I. scapularis* Salp15 or Salp15 Iric-1 (0–2000 ng/well) was added, incubated for 90 min, and washed, followed by incubation with anti-V5 antibody (Invitrogen) in 50 μL of blocking buffer for 45 min and 6 washes. Anti-IgG-HRP antibodies were incubated in 50 μL of blocking buffer for 45 min followed by 8 washes. Finally, tetramethylbenzidine substrate was added, the reaction was stopped, and absorbance was measured at 450 nm.

**Surface plasmon resonance (SPR).** The 3 OspCs were coupled to a CM5 sensor chip. A control channel on each sensor chip was coated with BSA or treated with ethanolamine, to determine the background binding signal. Specific binding of *I. scapularis* Salp15 and Salp15 Iric-1 to the different OspCs was corrected for nonspecific binding to the control channel. Different molar concentrations (range, 0–4500 nmol/L) of *I. scapularis* Salp15 and Salp15 Iric-1 in PBS–Tween 0.005% were injected for 300 s at a flow rate of 5 μL/min, and binding was monitored in real time. Regeneration of the sensor chip was achieved by a 30-min wash with a buffer containing 0.08 mol/L NaSCN, 0.31 mol/L MgCl₂, 0.15 mol/L urea, and 0.31 mol/L guanidine-HCl, followed by equilibration with PBS-Tween. Binding kinetics were analyzed with BIAevaluation software (version 3.2). Control experiments with recombinant *Escherichia coli*-expressed human tumor necrosis factor (TNF)–α with a C-terminal His-tag and a vehicle control were also performed.

**In vitro protection assays.** To each well (Greiner Bio 96 U-well plate), 50 μL of BSK-H medium containing 1 x 10⁸ spirochetes/mL and 4 μg of Salp15 (1μg/μl or a corresponding volume of PBS) were added. After 1 h, 46 μL of serum or antiserum (diluted in PBS) was added and incubated for 16 h at 33°C. Antiserum was derived from rabbits immunized with the indicated *Borrelia* species, and normal rabbit serum was derived from preimmune (naive) rabbits. Antisera recognized comparable antigen patterns (Supplemental figure 1), and titration experiments showed that the dilution of the antisera to reduce the numbers of viable spirochetes to approximately 30% was 1:200 for anti-N40 and 1:300 for anti-PBi and anti-pKo (data not shown). Normal rabbit serum was diluted accordingly. Experiments were performed in duplicate, a 25-μL aliquot from each well was diluted 5 times, and the number of live (i.e., motile) spirochetes was counted using dark-field microscopy (magnification, × 250; 10 random fields per well).
Chapter 5

**In vivo experiments.** The generation of immune mice has been described elsewhere [8]. Briefly, pathogen-free C3H/HeN mice (8 mice/group) were infected with *Borrelia* (1 x 10^5 spirochetes/mouse) by needle inoculation, tested for antibodies against *B. burgdorferi* by ELISA 2 weeks after infection, and treated with intraperitoneal ceftriaxone. Absence of spirochetal DNA was confirmed by PCR on tissue derived from ear biopsies. Mice were reinfected with the indicated *Borrelia* species (1 x 10^5 spirochetes/mouse) preincubated with Salp15 Iric-1 (30 μg/1 x 10^5 spirochetes) or BSA. Tissue samples were collected 14 days after (re-)infection.

**Quantitative PCR and histopathology.** DNA from murine tissues was obtained with the DNeasy Kit (Qiagen). Quantitative PCR detecting *Borrelia flaB* and the gene for mouse β-actin (*Actb*) was performed as described elsewhere [8, 13]. Standards consisted of dilutions of genomic DNA from the indicated *Borrelia* species or mouse *Actb* (252 bp) cloned into the PCR2.1-TOPO vector (Invitrogen) as described elsewhere [13]. Histopathological changes in tibiotarsi and heart tissue were assessed as described elsewhere [13, 16, 17]. Infection appeared to be too short to induce arthritis, since no arthritis was observed (data not shown). Also, 4-μm-thick paraffin-embedded sections of sagittally dissected hearts were processed and stained with hematoxylin-eosin by routine histological techniques. Carditis was scored by an independent pathologist blinded to the experimental design on a scale from 0 to 3 (0, no inflammation; 1, mild inflammation with <2 foci; 2, moderate inflammation with ≥2 foci; 3, severe inflammation with focal as well as diffuse inflammation covering a large area) [16, 17]. As described elsewhere, inflammatory infiltrates were localized almost exclusively at the basis of the aorta and the adjacent ventricular walls [18].

**Statistical analysis.** Differences between the groups were analyzed using 2-sided nonparametric tests, and, whenever appropriate, multiple-comparison correction was applied (version 4.0; GraphPad Prism Software). Data are presented as means ± SEs.

**Results**

**Identification and cloning of Salp15 Iric-1.** Recently we identified 3 homologues of *I. scapularis* Salp15 in salivary glands from fed adult *I. ricinus* ticks [11]. One of these homologues shared 82% homology with *I. scapularis* Salp15 at the DNA level and was designated Salp15 Iric-1. To assess the role played by the Salp15 homologues in *I. ricinus* in the pathogenesis of Lyme borreliosis, we first defined their relative expression in salivary glands 3 days after engorgement (figure 1A). Both Salp-15 Iric-1 and Iric-2 were shown to be highly expressed. Because Salp15 Iric-1 was most similar to *I. scapularis* Salp15, we expressed this protein in a *Drosophila* expression system (figure 1B). Salp15 Iric-1 and *I. scapularis* Salp15 consist of 134 and 135 aa, respectively. However, the apparent molecular weight of Salp15 Iric-1 is 5 kDa greater than that of *I. scapularis* Salp15. Analysis of the amino acid sequences of the 2 proteins (http://www.cbs.dtu.dk/services/NetNGlyc) revealed that Salp15 Iric-1 has 4 and that *I. scapularis* Salp15 has 2 predicted N-glycosylation sites (figure...
Preferential protection of Borrelia burgdorferi by Ixodes ricinus Salp15

Interaction of Salp15 Iric-1 with B. burgdorferi sensu stricto. We first demonstrated that, similar to I. scapularis Salp15, Salp15 Iric-1 binds to OspC in lysates from B. burgdorferi strain N40 in a solid-phase overlay (figure 2A). Salp15 Iric-1 also bound to OspC from B. burgdorferi strain B31 (Supplemental figure 2) but did not bind to a mutant strain lacking OspC (figure 2A). In addition, when overlays were performed with vehicle control instead of Salp15 proteins, similar background
Figure 2. Interaction of Salp15 Iric-1 with Borrelia burgdorferi strain N40. A, Solid-phase overlays with B. burgdorferi strain N40 and B. burgdorferi strain 297 deficient for ospC for Ixodes scapularis Salp15 and Salp15 Iric-1. The left panel shows Coomassie blue staining of lysates from B. burgdorferi strain N40 (7.2 μg) and strain 297 deficient for ospC (5.1 μg), separated by SDS-PAGE and blotted onto an Immobilon-P membrane. The molecular weight (MW) was determined using a molecular weight marker. Western blot analysis using a monoclonal antibody, L22 1F8, recognizing outer surface protein (Osp) C from B. burgdorferi sensu lato confirmed that the 22-kDa band was indeed OspC. Binding of recombinant I. scapularis Salp15 or Salp15 Iric-1 to native OspC was assessed by a solid-phase overlay with 1 μg/mL I. scapularis Salp15, 1 μg/mL Salp15 Iric-1, or vehicle control as a negative control, and binding was visualized using mouse anti-V5 and rabbit anti–mouse IgG–horseradish peroxidase (HRP) antibodies. B, Purified Escherichia coli–expressed recombinant OspC from B. burgdorferi strain N40, separated by SDS-PAGE and visualized by Coomassie blue staining. Lane 1, Molecular weight marker; lane 2, N40-OspC. C, OspC-Salp15 ELISA. N40-OspC (400 ng/well) was coated onto ELISA plates and incubated with different concentrations of I. scapularis Salp15 (black line) and Salp15 Iric-1 (gray line). Binding was visualized by incubation with a secondary and tertiary antibody, anti-V5-HRP and anti–mouse IgG–HRP antibodies, respectively. After addition of the substrate, absorbance was read at 450 nm. As a control, bovine serum albumin (BSA) was coated, and background signals did not exceed the indicated dashed line. This graph is representative of 5 independent experiments. Error bars represent SEs of triplicates from a single experiment. D, Surface plasmon resonance N40-OspC and I. scapularis Salp15 and Salp15 Iric-1. N40-OspC was bound to a CM5 sensor chip and different molar concentrations (ranging from 0 to 4500 nmol/L, represented by individual lines, with the highest line representing 4500 and the lowest line representing 0 nmol/L) of I. scapularis Salp-15 or Salp15 Iric-1 were injected for 300 s. Resonance units (RUs) depicted are after subtraction of the RUs from the BSA reference channel. Binding constants were calculated by 1:1 Langmuir fitting and confirmed by steady-state affinity plotting (see table 2). In addition, E. coli–expressed tumor necrosis factor–α with a C-terminal His tag and vehicle control did not show any binding (data not shown). E, In vitro protection assay in which spirochetes were preincubated with I. scapularis Salp15, Salp15 Iric-1 (final concentration, 40 μg/mL), or BSA as a control and then subjected to serum (diluted 1:200) from an N40-immunized rabbit (α-N40) for 16 h. Viable spirochetes were visualized by dark-field microscopy. Bars represent means ± SEs for 3 independent exper-
signals were observed compared with the overlays performed with the Salp15 proteins, further indicating that the binding was specific to OspC (figure 2A). To characterize further the binding of Salp15 Iric-1 to *B. burgdorferi* OspC, we generated soluble recombinant OspC derived from *B. burgdorferi* strain N40 (N40-OspC) (figure 2B) and performed an OspC-Salp15 ELISA. Similar to *I. scapularis* Salp15, Salp15 Iric-1 bound to plate-bound N40-OspC (figure 2C). To determine more specifically the affinity of the 2 proteins for N40-OspC, we performed SPR and demonstrated that the binding constant for Salp15 Iric-1 and N40-OspC was 2.23 x 10^-6 ± 0.15 x 10^-6 mol/L; for *I. scapularis* Salp15 and N40-OspC it was 0.78 x 10^-6 ± 0.14 x 10^-6 mol/L (figure 2D and table 2). Control runs with vehicle control or equimolar amount of recombinant TNF-α with a C-terminal His-tag did not show any binding (data not shown). To investigate whether the subtle difference in affinity had implications for the extent of protection from antibody-mediated killing, we performed in vitro protection assays with rabbit N40-immune serum. N40 spirochetes preincubated with either *I. scapularis* Salp15 or Salp15 Iric-1 appeared to be equally protected from N40-specific antibodies (figure 2E), also when lower concentrations of the Salp15 proteins were used (Supplemental figure 3). *I. scapularis* Salp15 has also been shown to protect *B. burgdorferi* strain N40 from antibody-mediated killing in vivo; N40 by itself could not reinfect N40-immune mice, but N40 preincubated with *I. scapularis* Salp15 could [8]. We performed a similar experiment with Salp15 Iric-1 and N40. N40-immune mice rechallenged with 1 x 10^6 N40 preincubated with 30 μg of *I. ricinus* were more readily reinfected (table 3) and were found to have significantly higher spirochete loads in several tissues 2 weeks after infection than N40-immune mice rechallenged with N40 preincubated with 30 μg of BSA (figure 2F), resulting in more severe carditis (figure 2G).

**Interaction of Salp15 Iric-1 with *B. garinii* and *B. afzelii***

In Europe, Lyme borreliosis is caused by 3 *Borrelia* species, *B. burgdorferi*, *B. garinii*, and *B. afzelii*. Therefore, we investigated the interaction of *B. garinii* strain PBi and *B. afzelii* strain pKo with Salp15 Iric-1. By solid-phase overlay, we demonstrated that Salp15 Iric-1 was able to bind to OspC from all 3 *Borrelia* species (figure 3A). To investigate the interaction further, we also produced soluble recombinant OspC derived from *B. garinii* strain PBi (PBi-OspC) and *B. afzelii* (pKo-OspC) (figure 3B). In both Salp15 Iric-1–OspC ELISA (figure 3C) and SPR (figure 3D and table 2), we showed that the affinity of Salp15 Iric-1 for OspC from PBi and pKo was comparable to the affinity of Salp15 Iric-1 for N40-OspC. Notably, Salp15 Iric-1 did not protect PBi and pKo from killing by specific antibodies in vitro (figure 3E), not even when higher amounts (i.e., 8 μg/well
**Table 2.** Binding kinetics of *I. scapularis* Salp15 and Salp15 Iric-1 for OspC from the different *B. burgdorferi* sensu lato strains.

<table>
<thead>
<tr>
<th>Salp15 protein</th>
<th>OspC</th>
<th>Ka (1/MS)</th>
<th>Kd (1/s)</th>
<th>KD (M)</th>
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<tr>
<td><em>I. scapularis</em> Salp15</td>
<td>N40-OspC</td>
<td>4,9E+03 ± 5</td>
<td>3,8E-03 ± 0,7E-03</td>
<td>0,78E-06 ± 0,14E-06</td>
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<tr>
<td>Salp15 Iric-1</td>
<td>N40-OspC</td>
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<td>10,2E-03 ± 2,5E-03</td>
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<tr>
<td>Salp15 Iric-1</td>
<td>PBi-OspC</td>
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<tr>
<td>Salp15 Iric-1</td>
<td>pKo-OspC</td>
<td>4,0E+03 ± 1,0E+03</td>
<td>9,7E-03 ± 2,5E-03</td>
<td>2,50E-06 ± 0,4E-06</td>
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</table>

**Note.** Binding curves of different molar concentrations of *I. scapularis* Salp15 and Salp15 Iric-1 to OspC from the different *Borrelia* species are shown in Figure 2 and 3. These data were analyzed with the BIA evaluation software (version 3.2) using 1:1 Langmuir fitting to calculate the KD. Results were confirmed by steady state affinity plotting (data not shown).

Ka: association constant, Kd: dissociation constant, KD: binding constant, data represent the mean of three independent experiments ± SE.

**Figure 3.** Interaction of Salp15 Iric-1 with *Borrelia garinii* strain PBi and *Borrelia afzelii* strain pKo. A. Solid-phase overlay with the different *Borrelia* species. Lane 0, molecular weight (MW) marker; lane 1, *Borrelia burgdorferi* strain N40; lane 2, *B. garinii* strain PBi; lane 3, *B. afzelii* strain pKo; lane 4, ospC-deficient strain 297. The amounts loaded on SDS-PAGE gel for *B. burgdorferi* strain N40, *B. garinii* strain PBi, *B. afzelii* strain pKo, and the outer surface protein (Osp) C mutant *B. burgdorferi* strain 297 were 7.2, 8.14, 8.62, and 5.1 μg, respectively. After transfer to an Immobilon-P membrane, Coomassie blue staining, Western blot, and solid-phase overlay using 1 μg/mL Salp15 Iric-1 were performed. B. Coomassie blue staining of recombinant N40-OspC (lane 2), PBi-OspC (lane 3), and pKo-OspC (lane 4). Lane 1, molecular weight marker. Western blot analysis using monoclonal antibody L22 1F8, recognizing OspC from *B. burgdorferi* sensu lato, confirmed that the 22-kDa recombinant proteins were indeed OspC. The affinity of Salp15 Iric-1 for the different OspCs was assessed by OspC ELISA (C) and by surface plasmon resonance.
Preferential protection of Borrelia burgdorferi by Ixodes ricinus Salp15

(D). E, In vitro protection assay in which N40, PBi, or pKo spirochetes were preincubated with Salp15 Iric-1 (final concentration, 40 μg/mL) or with bovine serum albumin (BSA) as a control and then subjected to serum from a rabbit immunized with the indicated Borrelia species for 16 h (anti-N40, diluted 1:200; anti-PBi and anti-pKo, diluted 1:300). Bars represent means ± SEs for 3 independent experiments. The average amount of viable spirochetes per dark-field microscope field in the well incubated with normal rabbit serum (NRS) was set at 100%. F, Borrelia burden determined by quantitative polymerase chain reaction in tissues from an in vivo experiment with B. afzelii strain pKo as described for N40 in the legend to figure 3. Eight to 9 mice per group were used. As a positive control, naive mice (n=3) were infected with pKo. These mice had high spirochete numbers in skin (688 ± 491 spirochetes/1 x 10^6 β-actin DNA copies), bladder (288 ± 100 spirochetes/1 x 10^6 β-actin DNA copies), and ankle tissue (1210 ± 813 spirochetes/1 x 10^6 β-actin DNA copies). These data are not depicted in the graphs, but the Y-axis of the graphs is based on these values. Both immune and naive mice were killed 2 weeks after (re)infection. G, Carditis severity scores in hematoxylin-eosin-stained sagittal sections of mouse hearts. In comparison, 3 naive mice infected with pKo and killed 2 weeks after infection had an average carditis score of 3 ± 0 (data not shown). A 2-sided Mann-Whitney U test was performed to determine statistical differences between the groups. * P < 0.05, ** P < 0.01, and *** P < 0.001. NS, not significant.

Salp15 Iric-1) were used (data not shown). In concordance with this finding, we demonstrated that the presence of Salp15 Iric-1 did not enhance the ability of pKo to reinfet pKo-immune mice (figure 3F and table 3), and there was no difference in carditis scores between pKo-immune mice rechallenged with strain pKo preinoculated with Salp15 Iric-1 and those rechallenged with strain pKo preinoculated with BSA (figure 3G). It proved not to be possible to reinfet PBi-immune mice with strain PBi, regardless of the presence or absence of Salp15 Iric-1, although the strain was clearly infectious in naive mice (table 3).

Table 3. PCR positivity of tissues from naive or immune mice.

<table>
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<th>Mice</th>
<th>Inoculum</th>
<th>Ear (pos/total)</th>
<th>Skin (pos/total)</th>
<th>Ankle (pos/total)</th>
<th>Bladder (pos/total)</th>
<th>Any organ (pos/total) and (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>N40</td>
<td>ND</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
<td>(9/9) 100%</td>
</tr>
<tr>
<td>Naive</td>
<td>pKo</td>
<td>ND</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
<td>(9/9) 100%</td>
</tr>
<tr>
<td>Naive</td>
<td>PBi</td>
<td>6/9</td>
<td>2/3</td>
<td>ND</td>
<td>2/3</td>
<td>(10/15) 66%</td>
</tr>
<tr>
<td>Immune</td>
<td>SHAM *</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>(0/8) 0%</td>
</tr>
<tr>
<td>Immune</td>
<td>N40 + BSA</td>
<td>ND</td>
<td>4/8</td>
<td>3/8</td>
<td>4/8</td>
<td>(11/24) 46%</td>
</tr>
<tr>
<td>Immune</td>
<td>N40 + Iric-1</td>
<td>ND</td>
<td>7/7</td>
<td>6/7</td>
<td>6/7</td>
<td>(19/21) 90%</td>
</tr>
<tr>
<td>Immune</td>
<td>pKo + BSA</td>
<td>ND</td>
<td>0/9</td>
<td>2/9</td>
<td>2/9</td>
<td>(4/27) 15%</td>
</tr>
<tr>
<td>Immune</td>
<td>pKo + Iric-1</td>
<td>ND</td>
<td>1/9</td>
<td>3/9</td>
<td>4/9</td>
<td>(8/27) 30%</td>
</tr>
<tr>
<td>Immune</td>
<td>PBi + BSA</td>
<td>ND</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>(0/24) 0%</td>
</tr>
<tr>
<td>Immune</td>
<td>PBi + Iric-1</td>
<td>ND</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>(0/24) 0%</td>
</tr>
</tbody>
</table>

Note. PCR positivity of tissues from naive or immune mice inoculated with 1 x 10^8 spirochetes. Tissue were analyzed two weeks post (re-)infection.

* This was demonstrated for N40-, PBi, and pKo-immune mice.

a In addition, in these mice low spirochetal numbers were detected by q-PCR (Fig 2).

b Statistically more tissues from N40-immune mice were re-infected with B. burgdorferi strain N40 in the presence of Salp15 Iric-1 compared to BSA, p=0.0018 (Two-sided chi-square test).

c This difference was not statistically significant for B. afzelii strain pKo.

d PBi-immune mice could not be re-infected with B. garinii strain PBi regardless of the presence or absence of Salp15 Iric-1, not even when a higher inoculum, 1 x 10^7 spirochetes was used (data not shown).
Discussion

The *I. scapularis* 15-kDa salivary protein Salp15 binds to OspC from *B. burgdorferi*, protecting the spirochete from antibody-mediated killing by the host [8]. Because we recently identified a Salp15 homologue, Salp15 Iric-1, in the European vector for Lyme borreliosis, *I. ricinus*, we hypothesized that this protein would also bind to OspC [11]. To test our hypothesis, we investigated whether Salp15 Iric-1 interacts with OspC from *Borrelia* strains representing the 3 major pathogenic *Borrelia* species in Europe. Therefore, we expressed Salp15 Iric-1 in a *Drosophila* expression system, similar to other tick proteins [19]; with SDS-PAGE, Salp15 Iric-1 appeared larger than the predicted molecular mass but also larger than *I. scapularis* Salp15. We demonstrated that these differences are due to differences in glycosylation.

Although the OspC sequences from the different *Borrelia* strains are only 70% homologous, Salp15 Iric-1 bound equally well to OspC from *B. burgdorferi* strain N40, *B. garinii* strain PBi, and *B. afzelii* strain pKo, as determined by ELISA and SPR. This suggests that the binding site of OspC for Salp15 Iric-1 is conserved between the 3 different *Borrelia* species. For ELISA and SPR we used truncated OspC, that is, mature OspC lacking the 20-aa leader sequence, because of unpublished observations indicating that *I. scapularis* Salp15 was unable to bind to full-length *B. burgdorferi* OspC. By SPR, we showed that *I. scapularis* Salp15 has a higher affinity for *B. burgdorferi* OspC than Salp15 Iric-1. The difference in affinity between the 2 proteins did not result in a difference in protection in the in vitro protection assays with *B. burgdorferi*. However, the amounts of Salp15 in both in vitro and in vivo experiments were based on previous research [8] and are estimated to be a surplus. We did not report the interaction between *I. scapularis* Salp15 and *B. garinii* or *B. afzelii* OspC because these spirochetes are not transmitted by *I. scapularis* in nature. Notably, the OspC sequence from *B. burgdorferi* strain N40 is 84% similar to the sequence of OspC from the *B. burgdorferi* reference strain B31, which is 100% identical to several European clinical isolates (PTa, Pho, Pboe, and PAIi).

Strikingly, Salp15 Iric-1 protected only *B. burgdorferi* strain N40 from antibody-mediated killing, resulting in significantly higher *B. burgdorferi* numbers and more severe carditis, compared with controls, in the in vivo experiments. Neither the *B. garinii* strain PBi nor the *B. afzelii* strain pKo was protected in the in vitro assays or the in vivo experiments with immune mice. This differential protection of *B. burgdorferi* by Salp15 Iric-1 could give *B. burgdorferi* a survival advantage over the 2 other *Borrelia* species in nature. Previously, we also demonstrated that *B. burgdorferi* outcompetes *B. garinii* in mice simultaneously inoculated with both *Borrelia* species through syringe inoculation [13].

The reason for the differential protection of *B. burgdorferi*, despite the ability of Salp15 Iric-1 to bind to OspC from all 3 pathogenic *Borrelia* species, can be explained in various ways. It could be that *B. burgdorferi* strain N40 expresses more OspC that the 2 European *Borrelia* strains. However,
we have previously shown that *B. afzelii* strain pKo expresses high amounts of OspC under in vitro conditions [20]. A different explanation would be that OspC, or the *I. scapularis* Salp15 or Salp15 Iric-1 binding site on OspC, is more exposed in *B. burgdorferi* than in *B. afzelii* and *B. garinii*. Whether this would be due to structural differences in the OspC proteins or to surrounding surface proteins remains an interesting topic for future research. Alternatively, the OspC surface consists of alternating positively and negatively charged areas that are important in the formation of OspC multimers or lattices on the surface of the spirochete [21]. Therefore, Salp15 binding to OspC could form a coating, which would explain the fact that the spirochete is protected not only from killing induced by anti-OspC antibodies but also from killing induced by *B. burgdorferi* antiserum [8]. Differences in OspC structure for *B. burgdorferi* compared with *B. garinii* and *B. afzelii* could affect the ability of the OspC dimers to form multimers or lattices and thereby expose other surface proteins, making the spirochete more susceptible to antibody-mediated killing. Last, it could be that other protein targets for bactericidal antibodies are more readily available in *B. garinii* or *B. afzelii*.

Interestingly, the OspC surface interface consists of a highly negatively charged cavity, suggested to be involved in protein-protein interactions [21, 22]. It is tempting to speculate that this site might be the binding site not only for Salp15 and Salp15 Iric-1 but also for the Salp15 paralogues in *I. ricinus*, Salp15 Iric-2 and -3. Therefore, if in vivo protection is dependent on the accessibility of this binding pocket, it seems unlikely that these Salp15 paralogues would protect *B. garinii* or *B. afzelii* against antibody-mediated killing. Another function of *I. scapularis* Salp15 is inhibition of CD4+ T cells by binding to CD4 [23–25]. It will be interesting to see whether the recently identified variants of Salp15 in *I. ricinus*, which share high sequence homology with *I. scapularis* Salp15 at the C-terminus, are able to inhibit T cell activation.

Currently, no vaccine is available to prevent *B. burgdorferi* sensu lato infection. Anti-tick vaccination strategies have been shown to prevent transmission of tick-borne pathogens [5]. Applying similar strategies to prevent Lyme borreliosis in Europe as well as in the United States is challenging because of the different *Ixodes* species that are the vectors for Lyme borreliosis. We have hypothesized elsewhere that Salp15 could serve as a candidate target [5, 11]. However, our present findings show that a Salp15 vaccine (targeting *I. scapularis* Salp15 as well as Salp15 Iric-1) would not be effective in Europe, because although it might prevent transmission of *B. burgdorferi* from the tick to the host, it probably would not prevent transmission of *B. afzelii* and *B. garinii*.

In summary, we have shown that a newly identified *I. ricinus* salivary protein, Salp15 Iric-1, binds to *B. burgdorferi* OspC, protecting the spirochete from antibody-mediated killing. In addition, we have demonstrated that Salp15 Iric-1 binds equally well to OspC from *B. garinii* and *B. afzelii* but that these *Borrelia* species are not protected. Our findings underscore the great complexity of tick-host-pathogen interactions in Lyme borreliosis in Europe.
Chapter 5

Acknowledgments

We are grateful to Dr. Utpal Pal and X. Frank Yang for the donation of the lysate from the ospC-deficient \textit{Borrelia burgdorferi} strain, Dr. Volker Fingerle (Max Planck Institute, Munich, Germany) for the donation of the anti-pKo and anti-PBi rabbit serum, and Dr. Martin Sprick (Academic Medical Center, Amsterdam) for the donation of the \textit{Escherichia coli}–expressed recombinant human His-tagged tumor necrosis factor–α. We also thank Robert Evers for his technical assistance with processing materials for histology.
Supporting information

**Supplemental figure 1.** *Borrelia* antigens recognized by rabbit antiserum. Shown are Western blot results for different antiserum from rabbits immunized with a specific *Borrelia burgdorferi* sensu lato strain. Lane 1, *B. burgdorferi* strain N40 lysate probed with anti-N40 rabbit serum; lane 2, *Borrelia garinii* strain PBi lysate probed with anti-PBi rabbit serum; lane 3, *Borrelia afzelii* strain pKo lysate probed with anti-pKo rabbit serum. The dilution of antiserum was 1:200, and the dilution of the secondary antibody (goat anti–rabbit–horseradish peroxidase) was 1:1000.

**Supplemental figure 2.** Binding of Salp15 Iric-1 to outer surface protein (Osp) C from *Borrelia burgdorferi* strain B31. Shown is a solid-phase overlay of *B. burgdorferi* sensu stricto strain B31 and Salp15 Iric-1. Lane 1, Coomassie staining of *B. burgdorferi* sensu stricto strain B31 lysate; lane 2, *B. burgdorferi* sensu stricto strain B31 lysate probed with 1 μg/mL Salp15 Iric-1. Binding of the tick protein to the *Borrelia* lysate was visualized using mouse anti-V5 antibodies and rabbit anti–mouse IgG–horseradish peroxidase antibodies, as described in figures 3 and 6.
Supplemental figure 3. Protection of *Borrelia* strains by Salp15 proteins at lower concentrations, as shown by protection assays using the different *Borrelia* strains. Spirochetes were incubated overnight with normal rabbit serum (NRS) or specific rabbit anti-*Borrelia* serum in the absence or presence of 20 μg/mL Salp15 Iric-1, *Ixodes scapularis* Salp15, or bovine serum albumin as a control in a total volume of 100 μL. Anti-N40 antiserum was diluted 1:200, whereas anti-PBi and anti-pKo were diluted 1:300. NRS was diluted accordingly. Viable spirochetes were enumerated by dark-field microscopy, as described in figures 3 and 6. A 2-sided Mann-Whitney U test was performed to determine statistical differences between the groups. \( P < .05. \)
References

24. Garg R, Juncadella IJ, Ramamoorthi N, et al. Cutting edge: CD4 is the receptor for the tick saliva immuno-