Tick-host-Borrelia interaction

*Implications for host immunity and vaccination strategies*

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Chapter 4

An *Ixodes ricinus* Tick Salivary Lectin Pathway Inhibitor protects *Borrelia burgdorferi* s.l. from human complement

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Introduction: We previously identified tick salivary lectin pathway inhibitor (TSLPI) in *Ixodes scapularis*, a vector for *Borrelia burgdorferi* sensu stricto (s.s.) in North America. TSLPI is a salivary protein facilitating *B. burgdorferi* s.s. transmission and acquisition by inhibiting the host lectin complement pathway through interference with mannose binding lectin (MBL) activity. Since *Ixodes ricinus* is the predominant vector for Lyme borreliosis in Europe and transmits several complement sensitive *B. burgdorferi* sensu lato (s.l.) strains, we aimed to identify, describe, and characterize the *I. ricinus* ortholog of TSLPI.

Methods: We performed (q)PCRs on *I. ricinus* salivary gland cDNA to identify a TSLPI ortholog. Next, we generated recombinant (r)TSLPI in a *Drosophila* expression system and examined inhibition of the MBL complement pathway and complement-mediated killing of *B. burgdorferi* s.l. in vitro.

Results: We identified a TSLPI ortholog in *I. ricinus* salivary glands with 93% homology at the RNA and 89% at the protein level compared to *I. scapularis* TSLPI, which was upregulated during tick feeding. In silico analysis revealed that TSLPI appears to be part of a larger family of *Ixodes* salivary proteins among which *I. persulcatus* basic tail salivary proteins and *I. scapularis* TSLPI and Salp14. *I. ricinus* rTSLPI inhibited the MBL complement pathway and protected *B. burgdorferi* s.s. and *Borrelia garinii* from complement-mediated killing.

Conclusion: We have identified a TSLPI ortholog, which protects *B. burgdorferi* s.l. from complement-mediated killing in *I. ricinus*, the major vector for tick-borne diseases in Europe.
Lyme borreliosis is a tick-borne disease caused by spirochetes of the *Borrelia burgdorferi* sensu lato (s.l.) complex that are transmitted by *Ixodes* ticks. In the USA, *B. burgdorferi* sensu stricto (s.s.) is the main Lyme borreliosis agent and is transmitted by *Ixodes scapularis* ticks in the Northeast and upper Midwest, and by *Ixodes pacificus* in the West Coast. In Western Europe, *Borrelia afzelii*, *Borrelia garinii*, and *B. burgdorferi* s.s. are the most common genospecies causing Lyme borreliosis and are transmitted by *Ixodes ricinus* ticks (Fingerle et al. 2008, Stanek et al. 2012). To enable tick feeding, ticks penetrate the skin with their hypostome and create a pool of uncoagulated blood. Ticks remain attached for multiple days and introduce saliva containing cement proteins, anticoagulants (Narasimhan et al. 2004, Hovius et al. 2008, Schuijt et al. 2013), immunosuppressive (Valenzuela et al. 2000, Ramamoorthi et al. 2005, Tyson et al. 2008) and other proteins that facilitate feeding. Moreover, various pathogens can be introduced through the saliva into the host skin where they can enjoy the benefits of these tick protein-induced effects (Kazimirova and Stibraniova 2013, Wikel 2013).

The classical, the lectin, and the alternative complement pathway are all involved in the host defence against *B. burgdorferi* s.l. (de Taeye et al. 2013). The lectin pathway is activated when mannose-binding lectin (MBL) or ficolins bind to carbohydrates on pathogen surfaces and activate MBL-Associated Serine Proteases (MASPs), leading to cleavage of C2 and C4 resulting in deposition of C3 convertase (C4bC2a). This will initiate the complement cascade, which triggers influx of immune effector cells, opsonophagocytosis, and direct lysis by membrane attack complexes (MAC) (Matsushita et al. 2013). Differences in complement sensitivity between *B. burgdorferi* s.l. genospecies are related to differential expression of complement regulating outer membrane proteins (Kraiczy et al. 2001, Kurtenbach et al. 2002, Bhide et al. 2005). In addition, *Ixodes* ticks introduce several salivary proteins, such as Salp15, Salp20, ISAC, and tick salivary lectin pathway inhibitor (TSLPI), which could give complement-sensitive *B. burgdorferi* s.l. strains a survival advantage during early skin invasion (Valenzuela et al. 2000, Daix et al. 2007, Schuijt et al. 2008, 2011a, Tyson et al. 2008, de Taeye et al. 2013).

We previously identified an *I. scapularis* protein, which was designated as TSLPI (GenBank acc. no. AEE89466.1), by screening an *I. scapularis* salivary gland protein library with tick-immune rabbit sera using yeast surface display technology (Schuijt et al. 2011b). TSLPI binds MBL and inhibits the host lectin complement pathway, mainly by inhibiting MBL-ligand binding. TSLPI plays a role in the transmission of *B. burgdorferi* s.s. from *I. scapularis* ticks to the murine host, as well as in acquisition from the host to larval *I. scapularis* ticks (Schuijt et al. 2011a). Moreover, silencing of
TSLPI and passive immunization with anti-TSLPI antibodies reduced *B. burgdorferi* s.s. loads in mice after infection by *I. scapularis* ticks, revealing its potential as an anti-tick vaccine candidate.

Because *I. ricinus* is the most important vector for *B. burgdorferi* s.l. in Europe, we examined the presence of an ortholog of *I. scapularis* TSLPI in *I. ricinus* and investigated its effect on the MBL complement pathway and complement-mediated killing of *B. burgdorferi* s.l.

**MATERIALS AND METHODS**

**Identification of TSLPI in *I. ricinus* salivary glands**

Fed *I. ricinus* salivary glands were collected from three adult female ticks laboratory-bred at Utrecht University, and RNA was isolated (Hovius et al. 2007) followed by a reverse transcriptase reaction, as described (Narasimhan et al. 2014). We designed primers A and B (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/vbz), of which primer A falls within the signal peptide of *I. scapularis* TSLPI (GenBank acc. no. HQ605983.1) and B falls within the 3’ end untranslated region. Using these primers, we performed a PCR using 2.5 μL *I. ricinus* SGE cDNA, 1 μL 10 mM primermix, 12.5 μL 2× ReddyMix (Thermo Scientific), and 8 μL of H₂O. PCR conditions were 95°C for 2 min, 25 cycles of 95°C 30 s, 58°C 30 s, 72°C 30 s, followed by 72°C for 10 min. DNA was extracted using a Wizard gel and PCR clean-up kit (Promega). The PCR product was ligated into a pGEM-T easy vector (Promega) and transformed into DH5-α cells (Invitrogen). Colonies were cultured in LB-ampicillin medium followed by plasmid isolation using a miniprep kit (Qiagen). Clones were sequenced using an automated sequencer (3730 DNA analyzer; AB Applied Biosystems). The sequence was submitted to GenBank under acc. no. KF451926.

**Quantitative PCR analysis of TSLPI expression during tick feeding**

*I. ricinus* nymphs were fed on a rabbit, and salivary glands of 50 nymphs were collected and pooled at 24 and 72 h of tick feeding. Salivary glands of unfed nymphs were also collected and pooled. RNA was isolated as described (Hovius et al. 2007) and purified using an RNeasy mini kit (Qiagen), and cDNA was produced as described above. A quantitative PCR (qPCR) for TSLPI mRNA expression was performed using primers C and D (Supplementary Table S1). *Ixodes ricinus* actin primers as described (Hovius et al. 2007) were used as a reference gene target for normalization. qPCRs
were performed using the LightCycler 480 (Roche) and SYBR green dye (Roche), and four replicates were performed for each. The PCR protocol was 95°C 6 min, and 50 cycles of 95°C 10 s, 60°C 20 s, and 72°C 20 s. Results were analyzed with LinRegPCR software (Amsterdam, The Netherlands) (Ruijter et al. 2009).

**In silico sequence analysis of *I. ricinus* TSLPI**

To assess the homology of the *I. ricinus* TSLPI mRNA sequence with *I. scapularis* TSLPI (salivary protein P8, AEE89466.1) and other known genes, we performed a Position-Specific Iterated Search (PSI-BLAST) through the NCBI website.

**Recombinant protein expression**

To produce glycosylated *I. ricinus* rTSLPI, the signal sequence was identified with Signalp 3.0 (Bendtsen et al. 2004) and PCR cloning with BGL-II and Xho1 restriction sites was performed, using Primers E and F (Supplementary Table S1) and High-Fidelity SuperMix (Invitrogen) under the following conditions: 30 s 94°C; 25 cycles of 30 s 94°C; 50 s 60°C; 25 s 72°C, followed by 5 min at 72°C. *I. ricinus* TSLPI was cloned into the pMT-Bip-V5-His tag vector (Invitrogen), which was transformed into *Drosophila* S2 cells together with pCO-Blast. Recombinant protein was expressed and purified using the Drosophila Expression System (Invitrogen) as described earlier (Schuijt et al. 2011b, 2013). After 8 weeks of blasticidin selection, the *Drosophila* cell culture was induced using copper sulfate for 3 days. The supernatant was filtered (0.45 μm) and run over a Ni-NTA column (Pierce) according to manufacturer’s protocols using 250 mM imidazole for elution. The elution was filtered (0.22 μm), concentrated with a 9 kDa protein concentrator (Pierce), and washed with sterile PBS three times to eliminate residual imidazole. The protein concentration was assessed by a DC-Protein-Assay (Bio-Rad). Purity was checked on 18% sodium dodecyl sulfate (SDS) gel using Coomassie blue staining.
MBL-dependent C4 deposition assay

High-binding microtiter plates (Microlon; Greiner) were coated with 10 μg/mL mannan (Sigma) in a coating buffer (15 mM Na2CO3, 35 mM NaHCO3, 15 mM NaN3; PH 9.6) overnight at 4°C. Wells were blocked using a blocking buffer (10 mM Tris-HCl, 145 mM NaCl, 15 mM NaN3, 1 mg/mL bovine serum albumin [BSA]) at room temperature (RT) for 2 h. After washing with TBS/0.05% Tween 20/5 mM CaCl2, normal human serum (NHS) diluted 512× was supplemented with I. ricinus TSLPI, I. scapularis TSLPI, or BSA diluted in C4 activation buffer (4 mM Na-diethyl-barbiturate, 145 mM NaCl, 2 mM CaCl2, 1 mM MgCl2, 0.02% Tween 20, 1 mg/mL BSA), and samples were incubated for 45 min at 37°C. MBL-deficient serum (<0.02 μg MBL/mL serum) with BSA was used as a negative control. Wells were washed and incubated with biotinylated monoclonal mouse anti-human C4 IgG (0.25 μg/mL) for 1 h at RT. After washing, streptavidin-conjugated horseradish peroxidase (HRP) diluted 1:10,000 in a washing buffer was incubated for 30 min at RT. After washing, 50 μL TMB substrate and 10 μL 3% H2O2 in NaAc buffer were added and absorbance was measured at 450 nm (optical density at 655 nm subtracted) using an iMark Microplate Reader (Bio-Rad).

Complement-mediated killing assay

We performed direct complement-mediated killing assays as described previously (Schuijt et al. 2011a, Wagemakers et al. 2014). Briefly, we added 0.1 to 1.0 μg/μL of I. ricinus rTSLPI or 1.0 μg/μL BSA to 25% NHS, incubated at 37°C for 30 min, and added this to 2.5 × 10^5 intermediate complement-sensitive (Kraiczy et al. 2000) B. burgdorferi N40 spirochetes grown to log-phase in a sterile V-shaped enzyme-linked immunosorbent assay (ELISA) plate (Greiner). Heat-inactivated human serum (HIS) (by incubation at 56°C for 30 min) was used as a control. The plate was incubated

Supplementary table S1. Primers used in this article

<table>
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<tr>
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<th>Primer Sequence</th>
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<tbody>
<tr>
<td>A</td>
<td>ATGGGGGTTGACTGAGACCAC</td>
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<tr>
<td>B</td>
<td>CTCCATTTTTTGCTCGGC</td>
</tr>
<tr>
<td>C</td>
<td>TGAGACAAATCATGGGACA</td>
</tr>
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<td>ATGGCATTCTCCGTTTTGAC</td>
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<td>CTAATCAGATCTCACAATTGCAC</td>
</tr>
<tr>
<td>F</td>
<td>GATATACTCGAGGAACTCAGTGCATTG</td>
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for 90 min, wells were resuspended, and 5 μL samples were examined by dark-field microscopy by a researcher blinded to the experimental design, and 100 spirochetes in each sample were scored as either motile or immotile. Complement-sensitive *B. garinii* strain A87S was incubated with 12.5% NHS or HIS, and samples were examined after 60 min of serum incubation.

**Statistics**
Data are represented as mean ± SEM. Comparisons were made using a Student’s *t*-test in GraphPad Prism 5.0, with Statistical significance: *p* ≤ 0.05 (*), *p* ≤ 0.01 (**), and *p* ≤ 0.001 (***)

**RESULTS**

*A TSLPI ortholog is expressed in I. ricinus ticks and is upregulated during tick feeding*

To identify a TSLPI ortholog, a PCR on *I. ricinus* salivary gland cDNA with primers based on the *I. scapularis* TSLPI signal sequence and 3′UTR resulted in a 315 bp product, which was ligated into a pGEMT-easy vector and eight clones were sequenced. The TSLPI sequence in *I. ricinus* was based on five identical clones and submitted to GenBank (acc. no. KF451926). Mature *I. ricinus* TSLPI is encoded by a 210 nucleotide coding region, which is 93% and 89% identical to *I. scapularis* TSLPI at the mRNA level and amino acid level, respectively. Next, we measured TSLPI mRNA expression in *I. ricinus* salivary glands during tick feeding to explore its significance in the feeding process. Similar to its ortholog in *I. scapularis* ticks (Schuijt et al. 2011a), *I. ricinus* TSLPI mRNA expression was not present in unfed nymphs and was upregulated during the course of feeding (Fig. 1A). In silico analysis using a PSI-BLAST revealed various homologous salivary proteins in *I. scapularis* and *I. pacificus* (Fig. 1B), including Salp9 putative anticoagulant (93% homology), anticoagulant Salp11 (90% homology), Salp14 (81% homology), and basic tail salivary proteins (BTSPs, e.g., 80% homology to BTSP-4). Notably, factor Xa inhibitor Salp14 also appears largely homologous to TSLPI, but has an additional C-terminal basic tail. It therefore appears that *I. ricinus* TSLPI is part of a larger family of proteins across multiple *Ixodes* species.
Figure 1. *Ixodes ricinus* expresses a TSLPI ortholog during tick feeding. (A) Quantitative PCR on TSLPI in salivary gland cDNA of *I. ricinus* nymphs collected at several time points during a bloodmeal. Nymphs were fed on a rabbit, and cDNA was pooled from 50 nymphs per time point. At $t=0$ (unfed ticks), no TSLPI expression was observed. Data represent mean ± SEM of four replicate samples. (B) Mature protein alignment of the *I. ricinus* TSLPI ortholog. SEM, standard error of the mean; TSLPI, tick salivary lectin pathway inhibitor.

**Functional characterization of *I. ricinus* TSLPI**

To investigate the function of *I. ricinus* TSLPI, we produced *I. ricinus* rTSLPI in a *Drosophila* expression system, resulting in a ~16 kDa protein (Fig. 2A). *I. ricinus* TSLPI showed a dose-dependent ability to inhibit MBL-dependent C4 activation, similar to its counterpart in *I. scapularis*, further confirming their functional homology (Fig. 2B). To investigate whether *I. ricinus* TSLPI inhibits complement-mediated killing of *B. burgdorferi* s.l., we performed in vitro killing assays in the presence of *I. ricinus* rTSLPI, in essence as previously described (Schuijt et al. 2011a). As expected, *I. ricinus* rTSLPI dose-dependently protected *B. burgdorferi* strain N40 from complement-mediated killing by NHS (25%) (Fig. 2C). Complement-mediated killing of the complement-sensitive strain *B. garinii* A87S by NHS (12.5%) was also diminished in the presence of *I. ricinus* TSLPI (Fig. 2D).
Figure 2. I. ricinus rTSLPI protects Borrelia burgdorferi s.l. from complement-mediated killing. (A) Coomassie blue stained 18% sodium dodecyl sulfate (SDS) gel, loaded with 2 μg of I. ricinus rTSLPI. (B) I. ricinus and Ixodes scapularis rTSLPI dose-dependently reduce MBL-dependent C4 deposition. An enzyme-linked immunosorbent assay (ELISA) plate was coated with mannan and incubated with NHS diluted 512× + rTSLPI at various concentrations. Serum MBL bound to mannan induced C4 deposition on the plates, which was detected using biotinylated mouse anti-human C4 antibody and developed with streptavidin-horseradish peroxidase (HRP). BSA incubated with NHS and BSA incubated with MBL-deficient human serum were used as a control. Data represent mean ± SEM. (C) Human serum complement-mediated killing of B. burgdorferi sensu stricto (s.s.) N40 in the presence of various I. ricinus rTSLPI concentrations. NHS in a final concentration of 25% was incubated for 30 min at 37°C with I. ricinus TSLPI at a concentration of 0.1, 0.5, or 1.0 μg/μL. In addition, 25% NHS +1 μg/μL BSA or 25% heat-inactivated human serum (HIS) +1 μg/μL BSA was used as controls. Spirochetes (2.5 × 10⁵) were added and incubated at 37°C. After 90 min, 100 spirochetes per well were scored (blinded) using dark-field microscopy as either motile or immotile. Results are representative of three independent experiments, and four biological replicates were used for each condition. Bars represent mean ± SEM. (D) Human serum complement-mediated killing of Borrelia garinii strain A87S in the presence of I. ricinus TSLPI. Spirochetes were incubated with 12.5% NHS for 60 min in the presence of I. ricinus rTSLPI or BSA (control) as described above. Representative of two independent experiments. BSA, bovine serum albumin; MBL, mannose-binding lectin; NHS, normal human serum. p ≤ 0.05 (*); p ≤ 0.01 (**), p ≤ 0.001 (***).
DISCUSSION

We have identified an ortholog of TSLPI in *I. ricinus* ticks and demonstrated that it is part of a larger family of *Ixodes* salivary proteins, comprising Salp9pac, Salp14, and BTSPs, among others. In addition, we showed that *I. ricinus* TSLPI is upregulated during *I. ricinus* tick feeding and that it inhibits the lectin pathway and thus protects both *B. garinii* and *B. burgdorferi* s.s. spirochetes from killing by the human complement system.

We showed for the first time that *I. ricinus* TSLPI aids *B. burgdorferi* s.l. to evade the host lectin complement system. Previously, other mechanisms have been described by which *I. ricinus* is able to inhibit the host complement system, namely by ISAC and IRAC, which inhibit the alternative pathway, and by Salp15, which by binding to its outer surface protects *B. burgdorferi* s.l. from MAC formation (Schroeder et al. 2007, Couvreur et al. 2008, Schuijt et al. 2008). In *I. scapularis* saliva, Salp15, Salp20, Isac, and TSLPI have been previously identified as anticomplement proteins (Valenzuela et al. 2000, Schuijt et al. 2008, 2011, Tyson et al. 2008). These proteins, in combination with its intrinsic complement evasion techniques, enable *B. burgdorferi* s.l. to evade killing by the host complement system (Pausa et al. 2003, Kraiczy et al. 2004, Pietikainen et al. 2010, de Taeye et al. 2013, Koenigs et al. 2013). Only two of the TSLPI/Salp14 family have been previously described in more detail; TSLPI (a lectin complement pathway inhibitor) (Schuijt et al. 2011a) and Salp14 (a factor Xa inhibitor) (Narasimhan et al. 2002, 2004). It is currently unknown whether TSLPI paralogs exert anticomplement activity. TSLPI, however, does not influence the coagulation cascade in contrast to Salp14, suggesting that the basic C-terminus of Salp14 is involved in factor Xa inhibition (data not shown). Moreover, the striking homology between TSLPI and several other *Ixodes* saliva proteins suggests that antibodies against TSLPI, which have previously been demonstrated to inhibit *B. burgdorferi* transmission by ticks, might also be directed against several of its paralogs. This mechanism might add to the effectiveness of TSLPI as the basis for an anti-tick vaccine candidate, and we are currently exploring different approaches to use TSLPI as an anti-tick vaccine.

CONCLUSIONS

*I. ricinus* ticks express TSLPI upon feeding, which is able to protect *B. burgdorferi* s.l. spirochetes from complement-mediated killing. TSLPI appears to be part of a larger protein family conserved among different *Ixodes* species.
ACKNOWLEDGEMENT

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.
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