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Coumou, J.

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Chapter 5

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

Alex Wagemakers, Jeroen Coumou, Tim J. Schuijt, Anneke Oei, Ard M. Nijhof, Cornelis van ’t Veer, Tom van der Poll, Adriaan D. Bins and Joppe W. Hovius

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Abstract

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 *I. 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. We performed a PCR 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*. 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. *I. ricinus* rTSLPI inhibited the MBL complement pathway and protected *B. burgdorferi* s.s. and *B. garinii* from complement-mediated killing. *In silico* analysis revealed that TSLPI appears to be part of a larger family of *Ixodes* salivary proteins resembling TSLPI and *I. scapularis* Salp14. In conclusion, we have identified a TSLPI ortholog which protects *B. burgdorferi* s.l. from complement-mediated killing in *Ixodes ricinus*, the major vector for tick-borne diseases in Europe.
Identification of a TSLPI ortholog in *Ixodes ricinus*

**Introduction**

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 *I. pacificus* in the West Coast. In Western Europe, *B. afzelii*, *B. garinii* and *B. burgdorferi* s.s. are the most common genospecies causing Lyme borreliosis, and are transmitted by *I. ricinus* ticks [80, 256]. 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 anti-coagulant [110, 178, 230], immunosuppressive [209, 278, 279] and other proteins that facilitate feeding. Moreover, various pathogens can be introduced via the saliva into the host skin where they can enjoy the benefits of these tick protein-induced effects [126, 293].

The classical, the lectin and the alternative complement pathway are all involved in the host defence against *B. burgdorferi* s.l. [62]. The lectin pathway is activated when MBL or ficolins bind to carbohydrates on pathogen surfaces and activate Mannose-binding lectin-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) [165]. Differences in complement sensitivity between *B. burgdorferi* s.l. genospecies are related to differential expression of complement regulating outer membrane proteins [19, 140, 143]. In addition, *Ixodes* ticks introduce several salivary proteins, such as Salp15, Salp20, ISAC and TSLPI, which could give complement-sensitive *B. burgdorferi* s.l. strains a survival advantage during early skin invasion [54, 62, 231, 232, 278, 279].

We previously identified an *I. scapularis* protein, which was designated as tick salivary lectin pathway inhibitor (TSLPI, Genbank accession AEE89466.1), by screening an *I. scapularis* salivary gland protein library with tick-immune rabbit sera using yeast surface display technology [234]. TSLPI binds Mannose Binding Lectin (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 [231]. 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.

Material 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 as described [111] followed by a reverse transcriptase (RT) reaction, as described [175]. We designed primers A and B (Supplemental data), of which primer A falls within the signal peptide of I. scapularis TSLPI (Genbank: HQ605983.1), and B falls within the 3' end untranslated region. Using these primers, we performed a polymerase chain reaction (PCR) using 2.5 µl I. ricinus SGE cDNA, 1 µl 10 mM primermix, 12.5 µl 2x Reddymix (Thermo Scientific, Waltham, MA, USA) and 8 µl of H2O. PCR conditions were 95 °C for 2 minutes, 25 cycles of 95 °C 30 sec, 58 °C 30 sec, 72 °C 30 sec, followed by 72 °C for 10 minutes. DNA was extracted using a Wizard gel and PCR clean-up kit (Promega, Madison, WI, USA) according to manufacturer's protocols using 250 mM NaHCO3, 15 mM NaN3; PH 9.6) overnight at 4 °C. Wells were blocked using mannan (Sigma, St. Louis, MO, USA) in coating buffer (15mM Na2CO3, 35mM NaHCO3, 15mM NaN3, 1 mg/ml BSA) at room temperature (RT) for 2 hours. After washing with TBS/0.05 % Tween-20/0.1 % BSA, plates were incubated with primary antibody (anti-TSLPI, Santa Cruz, CA) overnight at 4 °C. After washing with TBS/0.05 % Tween-20/0.1 % BSA, plates were incubated with HRP-conjugated secondary antibody (Jackson ImmunoResearch, PA, USA) for 1 hour at RT. Wells were washed and developed with TMB (United States Biochemical, Cleveland, OH, USA) for 15 minutes. Plates were then stopped with 2N H2SO4 and absorbance was measured at 450 nm.

Recombinant protein expression
To assess the homology of the TSLPI mRNA sequence with rTSLPI, the signal sequence was identified with Signalp 3.0 [17] and PCR cloning with BGL-II and Xho1 restriction sites was performed, using Primers E and F (Supplemental data) and High-Fidelity SuperMix (Invitrogen) under the following conditions: 30 sec 94 °C; 25 cycles of 30 sec 50 °C; 50 sec 60 °C; 50 sec 72 °C, followed by 5 minutes at 72 °C.

Identification of a TSLPI ortholog in I. ricinus
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.

qPCR 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 hours of tick feeding. Salivary glands of unfed nymphs were also collected and pooled. RNA was isolated as described [111] and purified using an RNeasy mini kit (Qiagen, Venlo, the Netherlands) and cDNA was produced as described above. A qPCR for TSLPI mRNA expression was performed using primers C and D (Supplemental data). Ixodes ricinus actin primers as described [111] were used as a reference gene target for normalization. qPCRs
were performed using the LightCycler 480 (Roche, Nutley, NJ, USA) 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) [222].

**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) via the NCBI website.

**Recombinant protein expression**

In order to produce glycosylated I. ricinus rTSLPI, the signal sequence was identified with Signalp 3.0 [17] and PCR cloning with BGL-II and Xho1 restriction sites was performed, using Primers E and F (Supplemental data) and High-Fidelity SuperMix (Invitrogen) under the following conditions: 30 sec 94 °C; 25 cycles of 30 sec 94 °C; 50 sec 60 °C; 25 sec 72 °C, followed by 5 minutes at 72 °C. *Ixodes ricinus* TSLPI was cloned into the pMT-Bip-V5-His tag vector (Invitrogen, CA), which was transformed into *Drosophila* S2 cells together with pCO-Blast. Recombinant protein was expressed and purified using the Drosophila Expression System (Invitrogen, CA) as described earlier [230, 231]. After 8 weeks of blasticidin selection, the *Drosophila* cell culture was induced using Copper sulphate for three days. The supernatant was filtered (0.45 µm) and run over a Ni-NTA column (Pierce, Waltham, MA, USA) 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, Hercules, CA, USA). Purity was checked on 18 % 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, St. Louis, MO, USA) in coating buffer (15mM Na2CO3, 35mM NaHCO3, 15 mM NaN3; PH 9.6) overnight at 4 °C. Wells were blocked using blocking buffer (10 mM Tris-HCl, 145 mM NaCl, 15mM NaN3, 1 mg/ml BSA) at room temperature (RT) for 2 hours. After washing with TBS/ 0.05 % Tween-20/ 5mM CaCl2, normal human serum (NHS) diluted 512x was supplemented with *I. ricinus* TSLPI, *I. scapularis* TSLPI or BSA diluted in C4 activation buffer (4mM Na-diethyl-barbiturate, 145 mM NaCl, 2mM CaCl2, 1mM MgCl2, 0.02 % Tween-20, 1
mg/ml BSA), and samples were incubated for 45 minutes 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 hour at RT. After washing, streptavidin-conjugated HRP diluted 1:10.000 in washing buffer was incubated for 30 minutes at room temperature. After washing, 50 µl TMB substrate and 10 µl 3% H₂O₂ in NaAc buffer was added and absorbance was measured at 450 nm (OD 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 [231, 290]. 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 minutes, and added this to 2.5x10^5 intermediate complement-sensitive *B. burgdorferi* N40 spirochetes grown to log-phase in a sterile V-shaped ELISA plate (Greiner). Heat-inactivated human serum (HIS) (by incubation at 56 °C for 30 minutes) was used as a control. The plate was incubated for 90 minutes, 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 minutes 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

In order to identify a TSLPI orthologue, 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 (Accession number 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 [231], I. ricinus TSLPI mRNA expression was not present in unfed nymphs, and was upregulated during the course of feeding (Figure 1A). In silico analysis using a PSI-BLAST revealed various homologous salivary proteins in I. scapularis and I. pacificus (Figure 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.

**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 (Figure 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 (Figure 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 [231]. As expected, I. ricinus rTSLPI dose-dependently protected B. burgdorferi strain N40
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Figure 2. *I. ricinus* rTSLPI protects *B. burgdorferi* s.l from complement-mediated killing. A. Coomassie blue stained 18 % SDS gel, loaded with 2 µg of *I. ricinus* rTSLPI. B. *I. ricinus* and *I. scapularis* rTSLPI dose-dependently reduce MBL-dependent C4 deposition. An ELISA plate was coated with mannan, and incubated with NHS diluted 512x + 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-HRP. BSA incubated with NHS and BSA incubated with MBL deficient human serum was used as a control. Data represent mean ± S.E.M. C. Human serum complement-mediated killing of *B. burgdorferi* N40 in the presence of various *I. ricinus* TSLPI concentrations. Normal human serum (NHS) in a final concentration of 25 % was incubated for 30 minutes at 37 °C with *I. ricinus* TSLPI at a concentration of 0.1µg/µl, 0.5µg/µl or 1.0µg/µl. In addition, 25 % NHS + 1µg/µl bovine serum albumin (BSA) or 25 % heat-inactivated human serum was used as a control. Results are representative of three independent experiments, and four biological replicates were used for each condition. Bars represent mean ± S.E.M. D. Human serum complement-mediated killing of *B. garinii* strain A87S in the presence of *I. ricinus* TSLPI. Spirochetes were incubated with 12.5 % NHS for 60 minutes in the presence of *I. ricinus* rTSLPI or BSA (control) as described above. Representative of two independent experiments.

from complement-mediated killing by NHS (25 %) (Figure 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 (Figure 2D).
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 BTSP proteins, among others. In addition, we showed that *I. ricinus* TSLPI is upregulated during *I. ricinus* tick feeding, 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.s. from MAC formation [50, 229, 232]. In *I. scapularis* saliva, Salp15, Salp20, Isac and TSLPI have been previously identified as anti-complement proteins [231, 232, 278, 279]. These proteins, in combination with its intrinsic complement evasion techniques, enable *B. burgdorferi* s.l. to evade killing by the host complement system [62, 133, 136, 197, 202]. Only two of the TSLPI/Salp14 family have been previously described in more detail; TSLPI (a lectin complement pathway inhibitor) [231] and Salp14 (a factor Xa inhibitor) [177, 178]. It is currently unknown whether TSLPI paralogs exert anti-complement 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.

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