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Chapter 4

A tick mannose-binding lectin inhibits the vertebrate complement cascade to enhance transmission of the Lyme disease agent

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Abstract
The Lyme disease agent *Borrelia burgdorferi* is primarily transmitted to vertebrates by *Ixodes* ticks. The classical and alternative complement pathways are important in *Borrelia* eradication by the vertebrate host. We recently identified a tick salivary protein, designated P8, which reduced complement-mediated killing of *Borrelia*. We now discover that P8 interferes with the human lectin complement cascade, resulting in impaired neutrophil phagocytosis and chemotaxis and diminished *Borrelia* lysis. Therefore, P8 was renamed the tick salivary lectin pathway inhibitor (TSLPI). TSLPI-silenced ticks, or ticks exposed to TSLPI-immune mice, were hampered in *Borrelia* transmission. Moreover, *Borrelia* acquisition and persistence in tick midguts was impaired in ticks feeding on TSLPI-immunized, *B. burgdorferi*-infected mice. Together, our findings suggest an essential role for the lectin complement cascade in *Borrelia* eradication and demonstrate how a vector-borne pathogen co-opts a vector protein to facilitate early mammalian infection and vector colonization.
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Abstract

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Introduction

During the blood meal, *Ixodes* ticks can transmit various pathogens to the vertebrate host such as *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum*, *Babesia* species and selected flaviviruses, among other agents [71]. Ticks introduce salivary proteins at the tick bite site to suppress host defense mechanisms, in order to successfully obtain a blood meal. Several anticoagulant, anti-inflammatory, anti-complement and immunosuppressive tick salivary proteins have been identified [110], which could not only facilitate tick feeding, but also enhance *Borrelia* transmission to the mammalian host and *Borrelia* acquisition by the tick vector [233].

The complement cascade is a major part of the mammalian innate immune response consisting of the classical, the alternative and lectin pathway and serves as a functional bridge between innate and adaptive immune responses [215]. The most prominent role of the complement system is to recognize and clear invading pathogens. Activation of the complement cascade results in opsonisation of the invading microorganism leading to enhanced phagocytosis, leukocyte chemotaxis and direct killing of pathogens by formation of the membrane attack complex (MAC). Initiation of the classical pathway relies on C1q binding to pathogens, either directly or to antibody-opsonized pathogens. The lectin pathway is activated upon binding of mannose-binding lectin (MBL) or ficolins (FCNs) to highly glycosylated pathogen associated molecular patterns (PAMPs) on the surface of pathogens [118, 164]. Both MBL and FCNs form a complex with pro-enzymes of MBL-associated serine proteases (MASPs) in serum, which are activated upon binding of MBL and FCNs to pathogens. Activated MASP-2 activates the complement cascade by cleaving C4 and C2 to generate the C4b2a complex. The alternative pathway spontaneously and continuously converts C3 and its predominant role is amplification of complement activation initially triggered by the classical and/or lectin pathway [129].

Several tick salivary proteins from different tick species inhibit the host complement system. A well-characterized tick complement inhibitor is the C5 activation inhibitor from the soft tick *Ornithodoros moubata* OMCI that inhibits C5 activation by binding C5 and preventing its activation by C5 convertase and inhibited complement-mediated haemolytic activity and the development of pathological features in a rodent model for myasthenia gravis [82, 102]. Other tick complement inhibitors, including Isac, Irac-1 and -2 and Salp20, belong to the Isac
protein family and inhibit the alternative complement pathway by binding and displacing properdin and thereby inhibiting the formation of C3 convertase [277, 279]. Also for *B. burgdorferi* sensu lato the complement cascade is an important obstacle to overcome in order to establish an infection in the mammalian host. *B. burgdorferi* sensu lato isolates differ in their susceptibility to complement-dependent killing [283], by expressing proteins on their outer membrane, named Erps and CRASPs, also known as Csp’s, which can bind host complement regulators such as factor H (FH) and factor H-like proteins (FHL-1) and by expressing a CD59-like protein on their outer membrane [28, 197]. Interestingly, we have previously shown that inhibition of the complement system by tick salivary proteins might be instrumental for survival of *B. burgdorferi* sensu lato strains that do not have the ability to evade complement-dependent killing by themselves [232].

By probing an *I. scapularis* salivary gland yeast surface display library with tick-immune rabbit sera we have identified an *I. scapularis* salivary protein with a predicted molecular weight of 8 kDa, designated P8, and demonstrated that this protein has anti-complement activity [234]. We here report the molecular mechanism by which P8 – from here on referred to as Tick Salivary Lectin Pathway Inhibitor (TSLPI) – inhibits the human lectin complement pathway and reveals a crucial role of the lectin pathway in the host immune response against *Borrelia*.

**Material and methods**

**Ticks and animals**

*Ixodes scapularis* nymphs and larvae were obtained from a tick colony at the Connecticut Agricultural Experiment Station in New Haven CT, USA. Ticks were maintained at 23 °C and 85 % relative humidity under a 14 h light, 10 h dark photoperiod. *Borrelia* infected nymphs were generated by placing larvae on *B. burgdorferi* infected C3H mice and fed larvae were molted to nymphs. For active immunization studies, 6 week old inbred New Zealand white rabbits (Charles River Laboratories) were obtained and immunized as described before [234]. Preparation of *I. scapularis* nympha salivary gland extract (SGE) and adult saliva are described in Supplemental Experimental Procedures. For the passive immunization experiments 4–6 weeks old female C3H/HeJ mice (Jackson Laboratory) were used. The protocol for the use of mice and rabbits was reviewed and approved by the Yale Animal Care and Use Committee. The Animal Care and
Use Committee of the University of Amsterdam approved all animal experiments performed in Amsterdam and experiments have been conducted according to national guidelines.

**Recombinant salivary proteins**

Cloning and expression of *tslpi* and *p19* in the *Drosophila* Expression System (Invitrogen) and purification of recombinant protein was performed as described before [234]. See the Supplemental Experimental Procedures for more details. Deglycosylation of recombinant TSLPI with N-Glycosidase (PNGase) F (Sigma) was performed according to the manufacturer’s instructions.

**Tick RNA isolation and quantitative RT-PCR**

Ticks were allowed to feed for 24, 72 h or to repletion (between 80 and 96 h after initiation of tick feeding, ticks were monitored two times a day and recovered after detachment from the hosts) on experimental and control animals. Nymphs were dissected and salivary glands and midguts were pooled (3 ticks), homogenized and RNA was extracted using the RNeasy minikit (Qiagen, CA). DNA was removed by on-column DNase digestion. The same procedure was performed with unfed ticks. Total RNA was quantified (5–6 ng of total RNA) and the quality of the RNA was assessed (Nanodrop 2000c). cDNA was synthesized using the iScript RT-PCR kit (Biorad, CA). According to the manufacturer’s instructions 2 µl of cDNA synthesis reaction was analyzed by quantitative PCR for the expression of tick actin and *tslpi*, using the iQ Syber Green Supermix (Biorad, CA) on a MJ cycler (MJ Research, CA) and previously described primers [234].

**Assay for detection of complement-mediated killing of Borrelia spirochetes**

The serum-sensitive *Borrelia garinii* isolate A87S was used (10^7 spirochetes ml⁻¹) to determine complement-mediated killing in normal human serum (NHS) as described earlier [232]. The serum resistant *Borrelia burgdorferi* N40 was used (10^7 spirochetes ml⁻¹) to determine antibody dependent complement-mediated killing in *Borrelia* immune human serum (IHS). See Supplemental Experimental Procedures for more details.

**Deposition of terminal C5b-9 complement complexes**

For detection of the terminal C5b-9 complement complexes on the Borrelian surface, a previously described C5b-9 immunofluorescence assay was used [232]. See Supplemental Experimental Procedures for more details.
Phagocytosis assay
Borrelia phagocytosis assay was performed as described before [109]. Experiments with human PMNs were performed with minor modifications described in Supplemental Experimental Procedures.

Neutrophil Migration Assay
Human PMNs were isolated from blood with Polymorph prep (Axis-Shield) according to the manufacturer’s instructions. PMN migration was assessed as previously described [109]. See Supplemental Experimental Procedures for more details. 12.5 % NHS was preincubated with 0.5 µg/µl BSA or rTSLPI for 30 min. Subsequently, 2.5 × 10⁷ B. garinii A87S was added and incubated for 30 min at 37 °C. Spirochetes were pelleted (30 min, 20,000 g) and the supernatant was checked for the absence of Borrelia using dark field microscopy. Supernatant was used to dilute the NHS to a final concentration of 2.5 % and was added to the bottom wells after which cell migration was assessed.

Borrelia binding assay
B. burgdorferi N40 (2.5 × 10⁷) was incubated with 0.1 ng/µl recombinant Salp15 or TSLPI in PBS/0.1 % BSA for 1 h at room temperature. Borrelia was pelleted and the pellet and supernatant were separated. The pellet was washed two times in 1.5 ml PBS/0.1 % BSA and was resuspended in the same volume as the supernatant. Equal volumes of supernatant and pellet was used to run on a SDS gel and transferred to nitrocellulose membranes. The membranes were blocked with PBS/5 % milk and immunoblots were probed and visualized with an HRP conjugated anti-V5 antibody (Invitrogen) and the enhanced chemiluminescence Western Blotting Detection System (GE Healthcare, NJ).

Classical and alternative complement pathway assays
Complement hemolytic activity of the classical pathway (CH50) was determined by using antibody coated sheep red blood cells (SRBCs). Opsonized SRBCs were incubated for 1 h with several dilutions of NHS preincubated with either 0.25 µg/µl neutralizing C3 antibody (antiC3-2, Sanquin), 50 µg/µl neutralizing C1q antibody (anti-C1q-85, Sanquin), 0.25 µg/µl BSA or 0.25 µg/µl rTSLPI in GVBS (VBS with 5.8 % (w/v) sucrose, 0.5 % (w/v) human serum albumin, 10 mM CaCl₂, and 2 mM MgCl₂, pH 7.4) in a shaker at 37 °C. Complement hemolytic activity of the alternative pathway (AP50) was performed by using rabbit red blood cells (RRBCs). RRBCs were incubated for 1 h in a shaker at 37 °C with several dilutions of serum preincubated with 0.25 µg/µl neutralizing C3 antibody, BSA or rTSLPI in GVBS containing 10 mM MgEGTA. For positive control of the CH50 and AP50 red blood cells were incubated in distilled water (100 % lysis of cells) and for the negative control the red blood cells were incubated in GVBS or GVBS with 10 mM EDTA, respectively (0 % lysis of cells). Plates were centrifuged at 1000 g for 5 min and hemolysis was determined by measuring OD at 414 nm using the iMark Microplate Reader (Biorad).

Lectin pathway of complement C4 activation
High binding microtiter plates (Microlon, Greiner) were coated with 10 µg/ml mannan (Sigma) or acetylated LDL (A-LDL) (Biomedical Technologies Inc.) in a moist chamber overnight at 4 °C. A-LDL is a useful ligand to specifically determine L-ficolin activity [75]. Wells were blocked with blocking buffer (1:10 dilution of Starting Block (Pierce, IL, US) in TBS with 0.05 % Tween20) at room temperature for 1 h. After washing with TBS/0.05 % Tween20/5 mM CaCl₂, several NHS dilutions preincubated for 30 min with either BSA, rP19 or rTSLPI were incubated in the wells in C4 activation buffer (4 mM Na-diethyl-barbiturate, 145 mM NaCl, 2 mM CaCl₂, 1 mM MgCl₂, 2 % (v/v) Starting Block, 0.02 % (v/v) Tween20) for 1 h at room temperature. Wells were washed and were incubated with biotinylated monoclonal mouse anti-human C4 IgG (0.25 µg/ml). After washing, avidin conjugated HRP in TBS/Tween20/Ca was incubated for 30 min at room temperature. Wells were washed, TMB substrate was added and the absorbance at 450 nm was measured using the iMark Microplate Reader (Biorad).

MBL binding
Wells (Microlon, HB, Greiner) were coated with 10 µg/ml mannan overnight at 4 °C, blocked with a 1:10 dilution of Starting Block (Pierce) in TBS with 0.05 % Tween20 and washed with TBS/Tween20/Ca. Several serum dilutions were incubated with BSA or rTSLPI for 30 min in MBL binding buffer (20 mM Tris-HCl, 1 M NaCl, 10 mM CaCl₂, 1 mg/ml BSA, 0.05 % (v/v) Triton X-100) and serum MBL was allowed to bind the mannan coated wells overnight at 4 °C. Wells were washed with TBS/Tween20/Ca and incubated with monoclonal mouse anti-human 10 µg/ml MBL antibody (Sanquin) in TBS/Tween20/Ca for 1 h at room temperature. After washing HRP-conjugated anti-mouse IgG was added and was incubated for 45 min at room temperature. The amount of MBL bound to the plates was determined by reading the absorbance at 450 nm using the iMark Microplate Reader (Biorad).
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GVBS containing 10 mM MgEGTA. For positive control of the CH50 and AP50 red blood cells were incubated in distilled water (100 % lysis of cells) and for the negative control the red blood cells were incubated in GVBS or GVBS with 10 mM EDTA, respectively (0 % lysis of cells). Plates were centrifuged at 1000g for 5 min and hemolysis was determined by measuring OD at 414 nm using the iMark Microplate Reader (Biorad).

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**MBL/MASP-2 complex activity assay**

The MBL/MASP-2 complex activity assay was used to determine C4 cleavage activity of MASP-2 as previously described [83]. Serum MBL/MASP-2 complexes were bound to mannan-coated plates as described above by using several serum dilutions (1:50, 1:100 and 1:300) in MBL binding buffer overnight at 4 °C. After washing the plates MBL deficient serum was used as a C4 source in a final dilution of 1:100 and incubated with 0.25 µg/µl rTSLPI, BSA or C1 inhibitor (Sanquin) in C4 dilution buffer for 1 h at room temperature. C4 activation and deposition on the plates as well as the amount of MBL bound on the plates after incubation of BSA or rTSLPI was determined as described above.

**Passive immunization and B. burgdorferi transmission**

In experiments to address Borrelia transmission to mice, six B. burgdorferi N40 infected nymphs were placed on each mouse passively immunized by intraperitoneal inoculation with 200 µl serum obtained from rTSLPI or OVA immunized rabbits. Nymphs were allowed to feed to repletion. Salivary glands and midguts were dissected for mRNA purification as described above. Transmission was assessed by quantitative PCR of DNA isolated from skin at 7 days and hearts and joints at 21 days post engorgement. To address Borrelia acquisition, at least 30 pathogen free I. scapularis larvae were placed on each B. burgdorferi-infected rTSLPI immune mouse and allowed to feed to repletion. At least three mice were used in each experiment. Larvae were analyzed in pools of 5.

**RNAi silencing of tslpi in Borrelia infected I. scapularis nymphs**

Primers were designed by addition of a T7 promoter site (TAATACGACTCATAAGGGAGA) at the 5’ end of the forward (AGGCTGCG-ACTATTACTGCTG) and reverse (TTGAATCGGTTGTCAAATGG) primers. dsRNA complementary to the tslpi gene was synthesized by using the MEGAscript RNAi kit (Ambion). The dsRNA was purified and quantified spectroscopically. dsRNA (5 nl, 1×10^{12} molecules per µl) was injected into the body of Borrelia infected nymphs using 10 µl microdispensers (Drummond Scientific, Broomall, PA) drawn to fine-point needles by using a micropipette puller (Sutter Instruments, Novato) or with the same volume of dsRNA elution buffer (mock), provided by the RNAi kit (Ambion) alone. The needles were loaded onto a micromanipulator (Narishige, Tokyo) connected to a Nanojet microinjector (Drummond Scientific). At least 50 nymphs were used in each group. The ticks were allowed to rest for 3 h before placement on mice. Ticks were allowed to feed for 72 h, were weighted and
dissected for mRNA isolation and quantitative RT-PCR as described above. *Borrelia* transmission was assessed by quantitative PCR of DNA isolated from skin at 7 days and hearts and joints at 21 days post engorgement.

**Statistical analysis**

The significance of the difference between the mean values of the groups was analyzed using a two-tailed student *t* test with Prism 5.0 software (GraphPad Software, USA) and the *p* value is indicated by asterisks in the figures.

**Results**

*Characterization of the Ixodes scapularis salivary protein TSLPI*

Recently, we identified an *I. scapularis* salivary protein - previously designated as P8 (GenBank: AEE89466.1) according to its molecular weight, but from here on referred to as Tick Salivary Lectin Pathway Inhibitor (TSLPI) based on its function - by probing a yeast surface display library with tick immune rabbit sera [234]. *I. scapularis* TSLPI has several homologues in *I. ricinus* (GenBank: AAS68352.1 and GenBank: AAP93884.1) indicating that this protein is conserved between these closely related *Ixodes* species, that both transmit *Borrelia*. Nucleotide homology between the genes was 83–86 % and amino acid similarity between the proteins was 84 %, with 73–75 % identity (Figure 1A). Although TSLPI is a member of a previously described group of anti-coagulant proteins [177], it did not show anti-coagulant activity [234]. *Drosophila*-expressed recombinant TSLPI was glycosylated and removing N-glycans from the protein backbone using N-glycosidase (PGNase) F reduced the molecular weight by 4 kDa (Figure 1B). Since TSLPI was antigenic in tick-immune rabbits, it is likely a secreted salivary protein [234]. Indeed, a signal peptide cleavage site was predicted, using the web-based software SignalP, between position 21 and 22 (Figure 1A) and native TSLPI was present in *I. scapularis* saliva when probed with rTSLPI antiserum (Figure 1C). In line with this, Ribeiro *et al* identified and annotated several proteins with high amino acid sequence identity to TSLPI (91–99 %) as secreted salivary proteins [214]. In *Borrelia*-free nymphs *tslpi* mRNA was upregulated during the course of the tick blood meal (Figure 1D). In the early phases of nymphal engorgement, *B. burgdorferi* infection of ticks resulted in significantly higher *tslpi* mRNA levels, whereas after blood feeding lower *tslpi* mRNA levels were found in *Borrelia*-infected ticks compared to *Borrelia*-free ticks (Figure 1D). *tslpi* mRNA was not detected in midguts of either *Borrelia*-free or *Borrelia*-infected nymphs at all tested time points of feeding
Figure 1. Characterization, protein sequence and expression of TSLPI. A. Multiple sequence alignment of *I. scapularis tslpi* aligned with two homologs of TSLPI in *I. ricinus*. Amino acids in white on a black background are identical; residues in white on a gray background are similar. Region inside the gray box shows the predicted signal peptide sequence. B. Coomassie staining of rTSLPI on SDS-PAGE before and after deglycosylation with PNGase F. C. *I. scapularis* saliva probed with rTSLPI-rabbit antiserum or OVA-rabbit antiserum. D. Expression profile of *tslpi* in salivary glands (SG) of pathogen-free (clean) nymphs and *Borrelia*-infected (Bb) nymphs./tslpi expression in unfed nymphs, after 24 and 72 hr of feeding, and repleted nymphs. Results represent mean ± SEM of values.

(data not shown), indicating that *tslpi* is preferentially expressed in *I. scapularis* salivary glands.

**TSLPI impairs direct killing of *B. burgdorferi sensu lato* isolates by the complement system**

We have previously shown that rTSLPI protects serum-sensitive *B. garinii* A87S against complement-mediated killing [234]. We here demonstrate that *B. garinii* A87S is protected from complement-dependent killing by rTSLPI in a dose-dependent manner, utilizing a *B. garinii* killing assay with 12.5 % normal human serum (Figure 2A; left panel). A growth inhibition assay confirmed the results of actual killing and the protective effect of rTSLPI against complement mediated killing of *Borrelia* (Figure S1A). *B. burgdorferi* N40, which is resistant to normal human serum (NHS) was killed by the complement system in the presence of *Borrelia*-opsonizing antibodies using 12.5 % *Borrelia*-immune human serum (IHS), (Figure 2A; right panel).
Lectin pathway and *Borrelia* survival

Figure 2. Recombinant protein TSLPI inhibits complement-mediated killing of *Borrelia* in a dose-dependent manner and inhibits *in vitro* chemotaxis and phagocytosis of *Borrelia* by PMNs. A. *B. garinii* strain A87S was incubated with 12.5% NHS (left), and *B. burgdorferi* strain N40 was incubated with 12.5% IHS (right) in the presence of increasing concentrations of BSA (control), control tick salivary protein rP19, or rTSLPI. As a control, spirochetes were also incubated with heat-inactivated (H) NHS or IHS. B and C. rTSLPI (B) or nymphal SGE (C) was preincubated with heat-inactivated OVA-rabbit antiserum (a-OVA) or rTSLPI-rabbit antiserum (a-rTSLPI). After 1.5 hr of incubation with serum, the percentages of immotile spirochetes were determined. Asterisks indicate a statistically significant
difference with the BSA control. D, C5b-9 deposition (red) on B. garinii A87S (green) incubated with 12.5 % NHS in the presence of 0.25 µg/µl BSA or rTSLPI for 15 min (upper) and for 30 min (lower). Right: Percentage of spirochetes with C5b-9 complexes. Results based on three independent experiments. E and F. Phagocytosis of CFSE-labeled viable B. garinii A87S (E) or B. burgdorferi N40 (F) by human PMNs in the presence of 1 % or 2.5 % NHS or 5 % IHS preincubated with 0.25 µg/µl BSA or rTSLPI at 37 °C. Cells were subjected to FACS analysis after 2, 5, 10, and 30 min of incubation. Heat-inactivated IHS and NHS were used as controls. Asterisks indicate a statistically significant difference with the BSA control. G. The chemoattractant capacity of NHS preincubated with 0.5 µg/µl BSA (control) or rTSLPI and 2.5 × 10^5 B. garinii A87S was assessed in a Transwell in vitro migration assay with human isolated PMNs. Fluorescence (fluorescent units, FU) measured at 1 min intervals at the bottom side of the well is shown and represents the number of PMNs migrated in time. Values represent the mean ± SEM.

Strikingly, recombinant TSLPI also protected B. burgdorferi N40 against antibody-mediated complement-dependent killing in a dose-dependent manner (Figure 2A; right panel). Heat-inactivated rTSLPI-rabbit antiserum completely abrogated the anti-complement activity of rTSLPI (Figure 2B), demonstrating the neutralizing capacity of these antibodies. In addition, heat-inactivated rTSLPI-rabbit antiserum substantially reduced the complement inhibitory activity of I. scapularis salivary gland extracts (Figure 2C), showing that TSLPI is a dominant complement inhibitor in tick saliva. To confirm that spirochetal killing by the complement system was induced by the formation of MAC complexes, deposition of C5b-9 was examined on the spirochete outer membrane. The amount of MAC complexes on the outer membrane of B. garinii A87S was greatly reduced when incubated with 12.5 % NHS for up to 30 min in the presence of 0.25 µg/µl rTSLPI (Figure 2D). Scanning electron microscopy demonstrated that the complement inhibitory effect of rTSLPI resulted in less bleb formation on the outer surface of Borrelia spirochetes (Figure S1B-C).

**TSLPI inhibits phagocytosis of B. burgdorferi sensu lato strains by human neutrophils and Borrelia-induced complement-mediated chemotaxis**

Activation of the complement system is important for recruitment of immune cells and phagocytosis. We therefore assessed whether rTSLPI impaired phagocytosis of Borrelia burgdorferi sensu lato isolates by human neutrophils. Flow cytometry showed that neutrophils were hampered in their ability to phagocytize B. garinii A87S in the presence of 0.25 µg/µl rTSLPI and either 1 % or 2.5 % NHS (Figure 2E). Human neutrophils also poorly phagocytized B. burgdorferi N40 in the presence of 0.25 µg/µl rTSLPI and 5 % IHS (Figure 2F). Complement splits products C5a and C3a, generated by the action of serine proteases, are potent chemoattractants for leukocytes [77]. Hence, next, the chemoattractant capacity of NHS pre-incubated with B. garinii A87S in the presence or absence of 0.5 µg/µl
rTSLPI was assessed in a Transwell in vitro migration assay with human PMNs (Figure 2G). As shown in Figure 2G fewer PMNs migrated towards NHS incubated with Borrelia and rTSLPI. Collectively, these data show that rTSLPI inhibits complement activation on Borrelia, resulting in impaired in vitro phagocytosis of Borrelia and impaired Borrelia-induced complement-mediated chemotaxis.

**TSLPI directly inhibits activation of the MBL complement pathway**

To understand how rTSLPI protected Borrelia against complement activation we next assessed whether complement inhibition by rTSLPI was Borrelia dependent or independent. We demonstrated that recombinant TSLPI did not bind Borrelia, as shown by a pull-down assay, suggesting that rTSLPI has a direct effect on the complement system (Figure 3A). Moreover, when rTSLPI was preincubated with 12.5 % NHS for 30 min, rather than added simultaneously with the spirochetes, B. garinii A87S was significantly more protected against complement-mediated killing.
(Figure 3B). A similar effect was observed when 12.5% IHS was preincubated with rTSLPI before it was added to *B. burgdorferi* (Figure 3B). To further elucidate the mechanism by which rTSLPI inhibited complement activation, we investigated the effect of rTSLPI on the classical, alternative and lectin pathway. Thus far, the classical and alternative complement pathways have shown to be important in *Borrelia* eradication by the host. Surprisingly, recombinant TSLPI did not inhibit lysis of antibody-sensitized sheep erythrocytes in a CH50 hemolytic assay, demonstrating that the classical complement pathway was not affected by rTSLPI (Figure 3C). Furthermore, the alternative pathway (AP50) was not inhibited by rTSLPI (Figure 3D). Since rTSLPI clearly inhibited complement mediated killing of *Borrelia*, the above negative results prompted us to investigate the effect of rTSLPI on the MBL complement pathway. Pre-incubation of serum with rTSLPI resulted in a pronounced dose-dependent inhibition of C4 deposition using mannan as a ligand, evidently showing that the MBL complement pathway was inhibited by rTSLPI (Figure 3E).

**The molecular mechanism by which TSLPI inhibits the MBL complement pathway**

We next set out to define the molecular mechanism by which rTSLPI inhibited the MBL complement pathway and assessed whether rTSLPI prevented binding of MBL to its ligands or inhibited complement activation through MASP-2 complement activation. Reduced MBL-ligand binding was observed when serum was pre-incubated with rTSLPI (Figure 4A, left panel) and increasing concentrations of rTSLPI dose-dependently decreased MBL binding to mannan (Figure 4A, right panel). In line with these observations MBL clearly bound to rTSLPI coated plates (Figure 4B, left panel), which did not result in activation of the MBL pathway (Figure 4B, right panel). Post-translational modifications, i.e. N-linked glycosylation, of recombinant Drosophila-expressed TSLPI, were shown to be crucial for its effect, since deglycosylation of rTSLPI by PGNase F largely abrogated the complement inhibitory effect of rTSLPI (Figure 4C), suggesting that TSLPI binds the carbohydrate recognition domains of MBL through its N-glycans. To rule out the possibility that rTSLPI could also impair MASP-2 activity, we investigated the effect of TSLPI on C4 deposition in a MBL/MASP complex activity assay. rTSLPI was incubated with bound MBL/MASP-2 complexes to determine the ability of activated MASP-2 to cleave complement C4. With comparable amounts of MBL/MASP-2 complexes (Figure S2) bound to mannan-coated plates, equal amounts of C4 deposition were measured after incubation with NHS in the presence of rTSLPI or controls (Figure 4D), demonstrating that rTSLPI does not
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A similar effect was observed when 12.5% IHS was preincubated with rTSLPI before it was added to B. burgdorferi (Figure 3B). To further elucidate the mechanism by which rTSLPI inhibited complement activation, we investigated the effect of rTSLPI on the classical, alternative and lectin pathway. Thus far, the classical and alternative complement pathways have shown to be important in Borrelia eradication by the host. Surprisingly, recombinant TSLPI did not inhibit lysis of antibody-sensitized sheep erythrocytes in a CH50 hemolytic assay, demonstrating that the classical complement pathway was not affected by rTSLPI (Figure 3C). Furthermore, the alternative pathway (AP50) was not inhibited by rTSLPI (Figure 3D). Since rTSLPI clearly inhibited complement mediated killing of Borrelia, the above negative results prompted us to investigate the effect of rTSLPI on the MBL complement pathway. Pre-incubation of serum with rTSLPI resulted in a pronounced dose-dependent inhibition of C4 deposition using mannan as a ligand, evidently showing that the MBL complement pathway was inhibited by rTSLPI (Figure 3E).

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Figure 4. Mechanism of the inhibiting effect of rTSLPI on the MBL pathway. A. Serum MBL binding to mannan coated plates was measured in the presence of rTSLPI. Left: serum was preincubated with 0.25 μg/μl rTSLPI or BSA or a neutralizing MBL antibody and MBL-deficient serum as a control. Right: MBL binding to mannan coated plates after preincubation of 1:50 NHS with several concentrations of BSA or rTSLPI. B. Serum MBL binding to rTSLPI or rP19 (control) coated plates (left), and complement activation was detected (right). C. C4 cleaving activity of the MBL lectin pathway in the presence of PGNase F-treated and untreated 0.25 μg/μl BSA or rTSLPI was determined using 1:40 NHS. D. The effect of rTSLPI on MASP-2 activity was determined by capturing MBL/MASP-2 complexes on mannan-coated plates by incubation of 1:50, 1:100, and 1:300 diluted serum in MBL-binding buffer. C4 deposition was measured in the presence of 0.25 μg/μl BSA, rTSLPI, or C1 inhibitor. E. Nymphal SGE was preincubated with heat-inactivated OVA-rabbit antiserum or rTSLPI-rabbit antiserum and was subjected to 1:40 NHS, and complement activation was measured on mannan-coated plates. Data are representative of two independent assays. Results are means ± SEM.
inhibit MASP-2 activity. Heat inactivated rTSLPI-rabbit antiserum reduced the lectin pathway inhibitory activity of *I. scapularis* salivary gland extracts (Figure 4E), showing that native TSLPI is a major inhibitor of complement activation through the lectin pathway.

Since the MBL-lectin pathway was specifically inhibited by rTSLPI, the effect of rTSLPI on complement-dependent killing was determined in MBL-deficient serum (<0.02 µg/ml MBL). Unexpectedly, rTSLPI significantly reduced complement-dependent killing of *B. garinii* A87S in MBL deficient serum (54.7 ± 0.88 % in the BSA control group to 31 ± 2.3% in the rTSLPI treated group; p=0.0007) (Figure 5A, right panel). In addition, when MBL activity was abrogated in NHS (MBL+/+) using a neutralizing MBL antibody, the percentage of non-motile spirochetes decreased from 81 ± 2.3% in the control group to 34 ± 1.8 % in the MBL antibody-treated group (p<0.0001) (Figure 5A, left panel). Interestingly, when the MBL antibody was combined with 0.25 µg/µl rTSLPI, spirochetal killing (20 ± 0.88 %) was significantly reduced compared to MBL antibody alone (p=0.002) (Figure 5A, left panel). These data suggest that rTSLPI inhibits additional factors besides MBL in the lectin pathway. It has been well-established that also ficolins are able to initiate the lectin pathway similarly to MBL. Therefore, we next assessed whether rTSLPI also influenced ficolin-induced C4 deposition. Faro *et al* previously described an assay to specifically measure L-ficolin binding and lectin pathway activation using acetylated low-density lipoprotein (Ac-LDL) as a ligand [75]. Indeed, rTSLPI dose-dependently impaired L-ficolin binding to Ac-LDL and thereby inhibited complement activation (Figure 5B).

**RNAi silencing of tslpi and TSLPI immunization impairs Borrelia survival**

Having established that TSLPI impaired the lectin pathway of complement activation and thereby impaired clearance of *Borrelia in vitro*, we set out to identify the role of TSLPI *in vivo*. We first demonstrated that rTSLPI also protected *Borrelia* against complement-dependent killing by normal mouse serum from C3H mice (Figure 6A). To assess the role of TSLPI in the transmission of *B. burgdorferi* from the tick to the host we injected *B. burgdorferi*-infected nymphs with dsRNA directed against *tslpi* (or mock injection as a control). Silencing resulted in a ~13 fold reduction of *tslpi* mRNA (Figure 6B). The *Borrelia* loads in nymphs (Figure 6C) 72 h post feeding were significantly reduced in *tslpi*-silenced *Borrelia*-infected nymphs, showing the importance of TSLPI for *Borrelia* persistence in the tick midguts during feeding.
Moreover, transmission of *Borrelia* was impaired from *tslpI*-silenced nymphs to mice, reflected by a significantly lower spirochete burden in skin after 7 days of infection, and in hearts and joints after 21 days of infection (Figure 6D). To further study the role of TSLPI in *Borrelia* transmission, mice were passively administered rTSLPI rabbit antiserum. The *Borrelia* load in skin from rTSLPI-immunized mice 7 days post infection was significantly lower and showed a trend towards lower *Borrelia* numbers in hearts and joints 21 days post infection (Figure 6E).

A strong innate immune response at the tick bite site, initiated by the host complement system, could also impair successful acquisition of *Borrelia* by *I. scapularis* from *Borrelia*-infected hosts. Therefore, we next assessed the role of TSLPI in *Borrelia* acquisition. The acquisition of *Borrelia* by larval *I. scapularis* ticks was impaired from rTSLPI-immunized *B. burgdorferi*-infected mice (Figure 6F). In line with this observation, larvae that molted to nymphs after feeding on rTSLPI-immunized *Borrelia*-infected mice had lower spirochetal loads (Figure 6G).

Together, these data show that TSLPI plays a significant role in both transmission of *Borrelia* from the arthropod vector to the naive mammalian host and acquisition of *Borrelia* from the infected mammalian host by larval ticks.
Figure 6. Decreased *Borrelia* survival after RNAi silencing of *tslpi* and in rTSLPI antiserum-immunized mice. A. *Borrelia* was incubated with 12.5% normal mouse serum (NMS) or with heat-inactivated (HI) NMS for 1.5 hr. B. *tslpi* expression in *B. burgdorferi* N40-infected nymphs after microinjection of TSLPI dsRNA or mock injection after 72 hr of feeding on mice. C. *Borrelia flaB* levels in nymphs in *tslpi*-silenced ticks and mock-injected ticks postfeeding on mice. D. *Borrelia* transmission from *tslpi*-silenced or mock-injected *Borrelia*-infected nymphs was determined by measuring *flaB* levels in mouse skin 7 days and in hearts and joint tissue 21 days postfeeding. E. *Borrelia* loads in murine skin 7 days and hearts and joints 21 days postfeeding on mice passively immunized with rabbit rTSLPI antiserum. F. *Borrelia* *flaB* levels in pooled larvae after feeding on mice passively immunized with rTSLPI antiserum or control rabbit serum. G. A second group of larvae were allowed to molt to the nymphal stage, and *Borrelia* *flaB* levels were determined. Horizontal bars represent the mean ± SEM. Asterisks indicate a statistically significant difference.
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Discussion

We here show that the lectin complement pathway is of paramount importance in the eradication of the causative agent of Lyme disease. Furthermore, we demonstrate how Borrelia co-opts a tick protein to inhibit the host complement system to facilitate early mammalian infection as well as vector colonization. Our findings add to the understanding of the molecular mechanisms exploited by B. burgdorferi s.l. to survive in its enzootic life cycle encompassing the mammalian host and the arthropod vector.

Until now, it has been shown that both the classical and alternative complement pathways are involved in complement-dependent killing of Borrelia [143, 283]. Interestingly, rTSLPI impaired complement-dependent killing of B. burgdorferi s.l. through specific inhibition of the lectin pathway, both in the absence or presence of specific antibodies, revealing a major role for the lectin pathway in eradication of Borrelia. MBL, the major player in the lectin complement pathway, is a C-type lectin that forms a non-covalent complex with MASPs and mainly circulates as a serum protein [118]. Via its carbohydrate recognition domains (CRDs), MBL recognizes and binds polysaccharide structures on various pathogens resulting in a conformational change causing auto activation of MASP-2 which subsequently initiates complement activation by cleavage of C4 and C2 [118]. We showed that rTSLPI did not affect MASP-2 activity, but prevented MBL binding to its ligand. Removing N-glycans from the protein backbone using PGNase F largely impaired the complement inhibitory effect of rTSLPI, suggesting that the glycosylated rTSLPI binds to the CRDs of MBL blocking downstream activation of the lectin pathway. Although activation of the lectin pathway is not antibody-dependent, rTSLPI protected serum resistant B. burgdorferi N40 when incubated with immune human serum. Anti-Borrelia antibodies play a crucial role in effective MAC formation in complement resistant strains by altering the outer membrane of Borrelia [132], which could explain the protective effect of rTSLPI in the presence of Borrelia antibodies. The importance of the MBL complement pathway in the direct lysis of Borrelia was underscored by the observation that a neutralizing MBL antibody greatly abrogated immobilization of B. garinii by MBL sufficient human serum. Approximately 25 % of the general population is MBL deficient, defined as a MBL serum level < 0.5 µg/ml [70], which could, based on our observations, make these individuals more susceptible for Lyme disease.
Until now MBL deficiency has been linked to several infectious diseases, such as malaria, HIV, meningococcal and pneumococcal disease among other diseases [208]. Complement-dependent killing of Borrelia in both NHS and MBL-deficient serum was completely abrogated by C1 esterase inhibitor (Figure S3), a serpin that inhibits both the classical pathway and lectin pathway of complement activation [215, 302], indicating that both the classical and lectin pathway are crucially important in initiating complement activation on Borrelia. In line with this finding, Borrelia complement-dependent killing was almost entirely ablated upon combined inhibition of the classical pathway and the lectin pathway by both neutralizing C1q antibody and rTSLPI (Figure S3). Irrespective of whether initiated by the lectin or classical complement pathway, C3 convertases bound to the surface of B. burgdorferi s.l. can induce amplification of the alternative pathway by enhancing C3 activation [215, 277], which could be detrimental for Borrelia survival. Recently, a constitutional role for MBL-associated serine protease 1 (MASP-1) in activation of the alternative pathway by cleaving factor D was demonstrated [272], showing that MASP-1 activation upon MBL and ficolin binding results in a direct activation of the alternative pathway as well. Thus, although the three complement routes are closely intertwined, our data demonstrate that the lectin pathway is of crucial importance in the eradication of Borrelia spirochetes.

Since rTSLPI also significantly reduced killing of Borrelia in MBL deficient serum and rTSLPI further reduced complement killing in normal serum incubated with neutralizing MBL antibodies, we assessed whether rTSLPI inhibited other components of the lectin pathway besides MBL. Apart from MBL, three types of ficolins, M-ficolin (also known as Ficolin-1), L-ficolin (also known as Ficolin-2) and H-ficolin (also known as Ficolin-3) are identified in humans and are able to activate the lectin pathway through the associated protease MASP-2 [164] and rTSLPI also showed to dose-dependently impair L-ficolin activity.

In addition to direct lysis of B. burgdorferi s.l. cells after MAC complex formation, complement activation also results in opsonisation of invading microorganisms and thereby enhancing phagocytosis by host immune cells [77, 215]. Indeed, opsonisation by complement factors was essential for phagocytosis of both B. garinii A87S and B. burgdorferi N40 by human PMNs, since these cells did not phagocytize Borrelia in the absence of a functional complement system, even in the presence of Borrelia-specific antibodies. Importantly, we observed that human
PMNs were significantly impaired in their capacity to phagocytize *B. garinii* A87S and *B. burgdorferi* N40 (data not shown) when normal human serum was preincubated with rTSLPI. Selected *Borrelia* strains are able to partially evade complement activation through the alternative pathway by interacting with host complement regulator proteins, such as factor H (FH) and factor H-like (FHL-1) through expression of Erps and CRASP proteins on their outer membrane [28] and by expressing a CD59-like protein that inhibits the assembly of (sub)lytic complexes [197]. However, others have shown that there was no difference in C3 deposition between serum sensitive strains and serum resistant strains when incubated in human serum [25, 132]. Since C3b deposition and its degradation products iC3b, C3c and C3d play a major role in complement receptor-induced phagocytosis [215], this indicates that, although serum resistant spirochetes evade direct lysis through the alternative complement route, they are equally susceptible to phagocytosis compared to serum sensitive *Borrelia*. In contrast, inhibition of complement initiating mechanisms on *Borrelia*, such as inhibition of the lectin pathway by TSLPI, is likely to result in decreased C3 opsonisation. This could explain the observed impaired phagocytosis of *Borrelia* by neutrophils incubated with normal human serum in the presence of rTSLPI. Interestingly, apart from initiating complement activation, MBL and ficolins also act as opsonins in the absence of C3 through several collectin receptors on phagocytic cells [118]. In addition, MBL has been shown to cooperate with TLR-2/TLR-6 to enhance phagosome signaling in response to bacteria [119], further underscoring the relevance of inhibition of the lectin pathway by TSLPI for *B. burgdorferi*.

Although rTSLPI inhibited *Borrelia*-induced MBL complement pathway-mediated chemotaxis *in vitro*, preliminary experiments indicate that rTSLPI did not impair influx of phagocytes in mice intradermally injected with $10^4$ *B. garinii* strain A87S (data not shown). It is possible that other tick proteins are necessary to impair innate immune responses towards *Borrelia*. Indeed, during tick feeding other salivary proteins are able to inhibit additional parts of the innate immune system such as dendritic cells, neutralizing chemokines, inhibition of inflammatory cytokines among other reported anti-inflammatory proteins [110], which could also play a role in protecting *Borrelia* from clearance by the host immune system.

tslpi was differentially expressed during the course of tick feeding. Interestingly, tslpi was upregulated in *Borrelia*-infected ticks during the early stages of tick feeding. Furthermore, the anti-complement effect of native TSLPI in tick
SGE was inhibited in both the *Borrelia* killing assay and lectin pathway assay in the presence of anti-rTSLPI antiserum, indicating that TSLPI is a major inhibitor of complement activation though the lectin pathway on *Borrelia* in *Ixodes* tick saliva and underscoring its biologic significance. Indeed, after silencing *tslpi* in ticks we showed that *Borrelia* transmission to mice was significantly impaired after 72 h of tick feeding. Moreover, *Borrelia* loads in ticks after feeding were also more than 2-fold reduced. Others have shown that complement is still active in midguts of *Ixodes* ticks post-engorgement [196]. Thus, our data suggest that *Borrelia* is protected against complement activation by TSLPI in midguts of ticks. We next extended our studies by passively transferring rTSLPI rabbit antiserum to mice. After 72 h of feeding on rTSLPI immunized mice spirochete transmission from *Borrelia*-infected ticks to mice was significantly reduced as well. Since immunization against rTSLPI did not result in total abrogation of *Borrelia* transmission from the tick to the host, a future tick antigen-based vaccine to prevent Lyme borreliosis should probably be based on a combination of tick proteins expressed during the course of tick feeding and important for *Borrelia* transmission [234]. Together, our findings implicate that TSLPI is crucially important for *Borrelia* survival both in the tick vector and in the host during early infection. While TSLPI directly and specifically inhibited the lectin pathway of complement activation, this could facilitate transmission of other pathogens as well. Although *tslpi* is specifically upregulated in tick salivary glands upon infection with *B. burgdorferi*, and not *Anaplasma phagocytophilum*, preliminary data show impaired *A. phagocytophilum* transmission by nymphs to rTSLPI-immune mice, using a mouse model for *A. phagocytophilum* infection (data not shown).

We also assessed the role of TSLPI in survival and migration of *Borrelia* from the mammalian host to ticks, a process referred to as acquisition. These experiments were performed with larval *I. scapularis* ticks since larvae naturally acquire *B. burgdorferi* s.l. and we have previously shown that *tslpi* is expressed in engorging larvae [234]. *Borrelia* acquisition from *Borrelia*-infected mice by larval ticks was impaired when mice were immunized with rTSLPI, indicating that TSLPI activity is crucial at the tick bite site for *Borrelia* in order to survive and migrate into the tick midgut as well. Thus, TSLPI is a tick salivary protein that plays a significant role in both *Borrelia* transmission to the mammalian host as well as *Borrelia* acquisition and persistence in ticks.
In conclusion, we have shown a crucial role for the lectin complement pathway in the eradication of the causative agent of Lyme disease. Furthermore, we have identified and characterized a tick salivary lectin pathway inhibitor (TSLPI) that abates complement activation on *B. burgdorferi* s.l. by impairing activation of the lectin complement pathway, which leads to impaired clearance of *Borrelia*. *Tslpi* silencing in ticks and TSLPI immunization studies demonstrated diminished *Borrelia* transmission and acquisition by ticks in which TSLPI activity was impaired, implying a crucial role for TSLPI in the enzootic life cycle of *B. burgdorferi* and revealing the potential of TSLPI as a component in a multivalent tick protein-based vaccine to prevent Lyme disease.
Supporting information

Figure S1. rTSLPI inhibits complement-mediated killing of *B. garinii A87S* by reducing bleb formation on the outer membrane of *Borrelia* spirochetes, related to Figure 2 A. *B. garinii A87S* was incubated with either normal human serum (15 %) in the presence of 0.5 µg/µl rTSLPI, BSA (control), 0.13 µg/µl rTSLPI or with 0.25 µg/µl C1-inhibitor (C1 inh) or with 15 % heat-inactivated serum. Growth of *Borrelia* was determined by measuring the color change of the indicator at 562/630 nm every 24 h for 144 h. Each sample and each time point were performed in triplicates and data are shown as mean ± SEM. B-C. rTSLPI reduced bleb formation on the outer membrane of *Borrelia* spirochetes. Normal human serum (12.5 %) was preincubated with 0.5 µg/µl rTSLPI (B) or BSA (C) and subsequently incubated for 2 hours with *B. garinii A87S*. Scanning electron microscope micrographs of *Borrelia* spirochetes in the (B) rTSLPI treated group and (C) BSA treated group. The white arrows indicate bleb formation on the outer surface of *Borrelia*.

Figure S2. rTSLPI does not influence MASP-2 activity, related to Figure 4D. MBL/MASP-2 complexes were captured on mannan coated plates by incubation of 1:50, 1:100 and 1:300 diluted serum in MBL-binding buffer. Serum was pre-incubated with 0.25 µg/µl rTSLPI, BSA (control) or C1 inhibitor. MBL deficient serum was used as a C4 donor and C4 deposition was measured in the presence of 0.25 µg/µl BSA, rTSLPI or C1 inhibitor as shown in Figure 4D. MBL binding to mannan coated plates was measured using an MBL antibody.
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Figure S3. Complement-mediated killing of *B. garinii* A87S in the presence of various inhibitors, related to Figure 5. Serum-sensitive *B. garinii* isolate A87S was incubated with 12.5% normal human MBL positive (1.16 µg/ml MBL; black bars) or MBL deficient (0.01 µg/ml MBL; grey bars) serum in the presence of 0.25 µg/µl BSA (control), 0.25 µg/µl rTSLPI, 15 µg/ml neutralizing MBL (anti-MBL) antibody, 0.1 µg/µl neutralizing C1q (antiC1q) antibody, 2 µg/µl C1 inhibitor single or a combination of inhibitors. As a control, spirochetes were also incubated with heat-inactivated (HI) NHS. Asterisks indicate a statistically significant difference with the control (***p<0.0001).

Supplemental Experimental Procedures

Saliva and salivary gland extract preparation. Adult tick saliva was collected and prepared as described previously [112]. Briefly, fully engorged *I. scapularis* adult female ticks were immobilized and a capillary tube was fitted over the mouthparts. 2 µl of 50 mg ml-1 of pilocarpine in ethanol was applied to the scutum of the ticks. Ticks were allowed to salivate for 3 hours and saliva was collected, pooled and stored at -80°C until use. Nymphal salivary gland extract was prepared by suspending and homogenizing one pair of salivary glands in 10 µl of PBS on ice. The homogenate was centrifuged (10000g for 10 minutes at 4°C) and supernatants were collected and pooled.

Production of recombinant salivary proteins. TSLPI and p19 cDNAs were inserted into the pMT/Bip/V5-HisA plasmid (Invitrogen, CA), and validated by sequencing. Drosophila melanogaster S2 cells were transfected with the plasmids containing TSLPI or p19 and stable transfectants were generated. Protein expression was induced with copper sulfate as described by the manufacturer (Invitrogen, CA). The supernatant was filtered using a 0.22-µm filter (Millipore, MA) and rTSLPI and rP19 were purified from the supernatant using NiNTA Superflow column chromatography (Qiagen, CA) and eluted with 250 mM imidazole. The eluted fractions were sterilized using a 0.22-µm filter, concentrated with a 5-kDa concentrator (Sigma-Aldrich, MO) and dialyzed against PBS. The purity of rTSLPI and rP19
was checked by Coomassie blue staining after electrophoreses on SDS 12 % polyacrylamide gel and the concentration was determined by BCA protein assay kit (Thermo Fisher Scientific inc., IL).

**Deposition of terminal C5b-9 complement complexes.** 12.5 % NHS was preincubated with either 0.25 µg/µl BSA or rTSLPI for 30 min at 33 °C. *B. garinii* strain A87S (2.5 x 10⁷) was incubated in NHS with BSA or rTSLPI for 15 or 30 min at 33 °C and were washed twice with PBS-1 % BSA. They were resuspended in PBS-1 % BSA, air dried on microscope slides overnight, and fixed in 100 % ice-cold acetone. Spirochetes were detected by incubation with *Borrelia* immune rabbit serum, and membrane bound C5b-9 complement complexes were labeled with monoclonal mouse C5b-9 antibodies (Dako). After being washed, the slides were incubated with an anti-rabbit immunoglobulin G-fluorescein isothiocyanate-labeled antibody (DAKO) and an anti-mouse immunoglobulin G-Alexa 546 (Invitrogen). Slides were mounted with Vectashield® mounting medium (Vector laboratories) and were visualized by confocal microscopy using a fluorescence microscope (Axiovert 200M; Carl Zeiss). At least 100 spirochetes were counted, and the experiment was performed three times.

**Assay for detection of complement-mediated killing of *Borrelia* spirochetes.** The serumsensitive *Borrelia garinii* isolate A87S was used (10⁷ spirochetes / mL) to determine complement-mediated killing in normal human serum (NHS). The serum resistant *Borrelia burgdorferi* N40 was used (10⁷ spirochetes / mL) to determine antibody dependent complement-mediated killing by *Borrelia* immune human serum (IHS). A final concentration of 12.5 % pooled NHS or IHS was pre-incubated with bovine serum albumin (BSA), rP19 or rTSLPI (0.25 µg/µl) respectively for 30 min at 33 °C and *Borrelia* (2.5 x 10⁷) was added. After 1.5 h of incubation at 33 °C spirochetes were examined by using dark field microscopy.

Serum samples were checked for *Borrelia*-specific antibodies by western blot analysis. Serum was heat inactivated by incubation at 56 °C for 30 minutes. *Borrelia*-cidal effect was recorded by screening for immobilization and bleb formation of the spirochetes (as shown in Figure S1B-C). Immotile spirochetes were considered dead, as described previously [283]. The percentages of non-viable spirochetes from 200 spirochetes per well were assessed [232]. As an alternative approach complement-mediated killing was assessed by a growth inhibition assay as previously described [137] with minor modifications. Briefly, *B. garinii* A87S (2 x 10⁷) were resuspended in 100 µl BSK-H medium containing 240 µg ml/L phenol red with 15 % normal human serum pre-incubated with 0.5 µg/µl rTSLPI or BSA, 0.13 µg/µl rTSLPI or with 0.25 µg/µl C1-inhibitor for 30 minutes at 33 °C. As a control *Borrelia* was resuspended in 100 µl BSK-H medium containing 240 µg / ml phenol red with 15 % heat-inactivated normal human serum. Samples were plated in 96 well microtiter plates (Microlon, Greiner) and incubated at 33 °C. Growth of *Borrelia* was determined by measuring the color change of the indicator at 562/630 nm each 24 h for 144 h using the Biotek Synergy HT plate reader.
Phagocytosis assay. Viable *B. burgdorferi* N40 or *B. garinii* A87S were labeled with 3.3 μM carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen). Human neutrophils (PMNs) derived from a blood donor were isolated with Polymorphprep (Axis-Shield) according to the manufacturer’s instructions. 1 % and 2.5 % NHS or 5 % IHS was preincubated with either 0.25 μg/μl BSA or rTSLPI for 30 min at room temperature. CFSE-labeled viable *Borrelia* were incubated with PMNs (PMN:*Borrelia* 1:25) and NHS or IHS was added to *B. garinii* or *B. burgdorferi* N40 respectively. Samples were incubated for 2, 5 and 10 min (NHS and IHS) or 30 min (IHS) on a shaker at 37 °C and phagocytosis was stopped by transferring the cells to ice-cold Fluorescence-Activated Cell Sorting (FACS) buffer (PBS supplemented with 0.5 % bovine serum albumin (BSA), 0.01 % NaN3 and 0.35 mM EDTA) and stored at 4°C. Samples continually kept on 4 °C were used as a control. Heat-inactivated serum (56 °C for 30 minutes) was used as a control to determine the role of serum complement in phagocytosis. Cells were washed with ice-cold FACS-buffer and incubated with BD Lyse/Fix solution (BD Biosciences) and resuspended in FACS-buffer for FACS analysis on a FACSCalibur flow cytometer (Beckton Dickinson). The phagocytosis index of each sample was calculated as mean fluorescence intensity (MFI)xpercentage positive cells at 37 °C minus the phagocytosis index at 4 °C. Each sample and each time point were performed in triplicates and were performed two times.

Neutrophil Migration Assay. Prior to experimentation human PMNs were labeled with 10 μM CellTracker Green (Molecular Probes) in serum-free Dulbecco's modified Eagle's medium (DMEM) for 1 h at 37 °C. The dye was fixed by 1 h incubation in DMEM plus 10 % FCS. Subsequently, PMNs were washed, resuspended in serum-free medium and transferred to 3 μM pore size HTS FluoroBlok Cell Culture Inserts (BD Falcon) which were inserted in fitting 24-well plates. 12.5 % NHS was preincubated with 0.5 μg/μl BSA or rTSLPI for 30 min. Subsequently, 2.5 x 10⁷ *B. garinii* A87S was added and incubated for 30 min at 37 °C. Spirochetes were pelleted (30 min, 20,000g) and the supernatant was checked for the absence of *Borrelia* using dark field microscopy. Supernatant was used to dilute the NHS to a final concentration of 2.5 % and was added to the bottom wells after which cell migration was assessed as previously described (Hovius et al., 2009). Briefly, fluorescence values representing the number of cells on the bottom side of the insert, was read during 30 cycles every 2 min on a Series 4000 CytoFluor Multi-Well Plate Reader (Perceptive Biosystems). Raw fluorescence data were corrected for background fluorescence and no-attractants controls were subtracted at each measured time point to correct for random migration. Migration start points were set to zero.