Tick-host-pathogen interactions in Lyme borreliosis
Hovius, J.W.R.

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The tick salivary protein Salp15 inhibits the killing of serum-sensitive *Borrelia burgdorferi* sensu lato isolates

Tim J. Schuijt,1 Joppe W. R. Hovius,2,3 Nathalie D. van Burgel,1 Nandhini Ramamoorthi,3 Erol Fikrig,3 and Alje P. van Dam1

1 Department of Medical Microbiology, Leiden University Medical Center, Leiden, The Netherlands 2 Center for Experimental and Molecular Medicine (CEMM), University of Amsterdam, AMC, Amsterdam, The Netherlands 3 Section of Infectious Diseases, Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut, USA
Abstract

*Borrelia burgdorferi*, the causative agent of Lyme disease, is transmitted by ticks. During transmission from the tick to the host, spirochetes are delivered with tick saliva, which contains the salivary protein Salp15. Salp15 has been shown to protect spirochetes against *B. burgdorferi*-specific antibodies. We now show that Salp15 from both *Ixodes ricinus* and *Ixodes scapularis* protects serum-sensitive isolates of *Borrelia burgdorferi* sensu lato against complement-mediated killing. *I. ricinus* Salp15 showed strong protective effects compared to those of *I. scapularis* Salp15. Deposition of terminal C5b to C9 complement complexes, part of the membrane attack complex, on the surface of *B. burgdorferi* was inhibited in the presence of Salp15. In the presence of normal human serum, serum-sensitive *Borrelia burgdorferi* requires protection against complement-mediated killing, which is provided, at least in part, by the binding to the tick salivary protein Salp15.
Introduction

The causative agent of Lyme disease, *Borrelia burgdorferi*, survives in a tick-mouse cycle. In the United States, *B. burgdorferi* sensu stricto is maintained primarily in *Ixodes scapularis* ticks, while the European vector of *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* strains are generally *Ixodes ricinus* ticks. Feeding of ixodid ticks normally takes several days (2), which gives the host immune system time to react to the arthropod. Ticks have developed several mechanisms to evade both innate and adaptive host responses, which enable them to take an effective blood meal. Tick saliva possesses proteins with immunosuppressive (14, 18), anticomplement (5, 19, 27), and antithrombotic (21, 22) activity. Salp15, a feeding-induced tick salivary protein, is known to inhibit CD4+ T-cell activation and proliferation by specifically binding to the CD4 co-receptor of the T cells (1, 6, 13). Also, Salp15 enhanced the survival of *B. burgdorferi* in the host after transmission by the tick by specifically interacting with *B. burgdorferi* outer surface protein C (OspC) and providing protection against borreliacidal antibodies (25). Recently we identified three Salp15 homologues in *I. ricinus* ticks (12), and one of these homologues, Salp15 Iric-1, showed 80% similarity to *I. scapularis* Salp15 (Iscap Salp15) at the DNA level.

The innate immune response, the complement system in particular, plays a crucial role in the eradication of invading pathogens. The complement system is also important in the initiation of an immune response against *B. burgdorferi*. The spirochetes are opsonized and also directly killed by the formation of the lytic pore-forming membrane attack complex (MAC) (3, 23). *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* isolates activate the complement cascade both by the classical pathway and by the alternative pathway in nonimmune human serum (NHS) in the absence of specific antibodies, but they differ in susceptibility to complement-mediated killing (28). Serum-resistant *Borrelia* strains are able to evade complement-mediated killing by binding to complement regulators of the alternative complement pathway, i.e., factor H and factor H-like protein-1 (FHL-1), through CRASP-1Bb (15), CRASP-2Bb (9), OspE (10), and/or CRASP-3Bb (16) proteins, or by expressing a CD59-like complement inhibitory molecule (24). The split products after complement activation are also important because of chemotaxis and the infiltration of immune cells in the *Borrelia*-infected tissue. Altogether, there are several reasons for the spirochetes to protect themselves against complement activation. In this study, we show that the tick salivary protein Salp15 plays a role in the protection of serum-sensitive *B. garinii* strains and intermediately resistant *B. burgdorferi* strains against direct killing by the complement system.

Materials and methods

*Borrelia* isolates and growth conditions.

Serum-sensitive strains *B. garinii* A87S and VSBP and intermediately resistant strains *B. burgdorferi* VS215 and B31 were used in this study. Both *B. garinii* strains are human isolates, while both *B. burgdorferi* strains are tick isolates. Spirochetes were cultivated at 33°C in Barbour-Stoenner-
Kelley medium plus sodium bicarbonate (BSK-H medium) supplemented with 6% rabbit serum (Sigma).

**Purification of recombinant *I. scapularis* and *I. ricinus* Salp15**

For the purification of Iscap Salp15 (GenBank accession number AAK97817), salp15 was cloned in frame in *Drosophila melanogaster* cells in conjunction with a His-tag, a V5 epitope, and a resistance gene for hygromycin as described previously (1). Salp15 Iric-1 (GenBank accession number ABU93613) was purified from *Drosophila* cells stably transfected with salp15 Iric-1 and a plasmid containing a blastomycin resistance gene (J. W. Hovius, T. J. Schuijt, K. A. de Groot, J. T. T. H. Roelofs, A. Oei, J. A. Marquart, C. van ’t Veer, T. van der Poll, N. Ramamoorthi, E. Fikrig, and A. P. van Dam, J. Infect. Dis. 2008;198: 1189). The Schneider *Drosophila* cells expressing the salp15 gene from *I. scapularis* or *I. ricinus* were selected with hygromycin (500 µg ml–1) or blastomycin (25 µg ml–1), respectively, and were grown in large spinner flasks together with penicillin and streptomycin (Invitrogen) for 3 days. *Drosophila* cells were subsequently induced with copper sulfate with a final concentration of 500 mM for 4 days and centrifuged at 1,000 x g for 15 min. The supernatant was filtered using a 0.22-µm filter (Millipore). Both Salp15 Iric-1 and Iscap Salp15 were purified from the supernatant by use of the HisTrap Ni²⁺ column (GE Healthcare) and eluted with 100 mM imidazole. The eluted fractions were filtered through a 0.22-µm filter and concentrated with a 5-kDa concentrator (Vivascience) through centrifugal concentration. The purity of the purified Salp15 was checked by silver staining (Bio-Rad) according to the manufacturer’s recommendations, and the concentration was determined with the Bradford assay.

**NHS**

Serum samples used were derived from one donor and were checked for the absence of antibodies against *B. burgdorferi* by Western blot analysis. Heat inactivation of NHS was achieved by incubation of the serum samples at 56°C for 30 min.

**Assays for detection of complement-mediated killing of spirochetes and Salp15 protection**

Two serum-sensitive *B. garinii* strains, the A87S and the VSBP strains, and two intermediately resistant *B. burgdorferi* sensu stricto strains, VS215 and B31, were used (10⁷ spirochetes ml–1). Spirochetes (2.5 x 10⁵) were preincubated with bovine serum albumin (BSA), Salp15 Iric-1, or Iscap Salp15 (80 µg/ml) for 30 min at 33°C. They were then incubated with NHS or heat-inactivated NHS and examined after 1.5 h, 4.5 h, and 24 h. The two parameters of borreliacidal effect that were recorded are immobilization and bleb formation of the spirochetes. Immotile spirochetes were considered dead (28). The percentages of immotile spirochetes for 200 spirochetes per well were assessed. In a separate titration experiment, different Salp15 concentrations, ranging from 5 µg/ml to 160 µg/ml, were also tested in the same way. To investigate whether membrane-bound Salp15 protects the spirochetes against antibody-independent complement-mediated killing, the spirochetes were washed twice with BSK-H medium (4,000 x g, 10 min) after incubation with
Salp15 Iric-1. After removal of unbound Salp15 by washing, the spirochetes were subjected to 12.5% NHS and examined for borreliacidal effect after 1.5 h, 4.5 h, and 24 h of incubation.

Subculture of *B. garinii* VSBP after incubation with *I. ricinus* Salp15

The serum-sensitive *B. garinii* VSBP strain was preincubated with Salp15 Iric-1 for 60 min at 33°C. Then, spirochetes were exposed to 50% NHS for 24 h. As described above, the surviving spirochetes were subcultured in BSK-H medium for 7 days. This selection process was repeated twice.

Binding of *I. ricinus* Salp15 to *B. garinii* VSBP in overlay assay

*B. garinii* VSBP lysates were obtained from 100-ml cultures that were grown to a density of 1 x 10⁸ spirochetes/ml. Lysates were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto an Immobilon-P membrane (Millipore) to have approximately similar amounts of OspC, as determined by Coomassie staining. After the membrane was incubated overnight in blocking buffer (1% BSA, 3% milk in TBS-0.05% Tween), it was incubated with 1 µg/ml purified Salp15 Iric-1 in blocking buffer for 1.5 h at room temperature, washed, and consequently incubated with a 1:5,000 dilution of horseradish peroxidase-conjugated V5 antibody (Invitrogen) in blocking buffer. Blots were developed by enhanced chemiluminescence.

Binding assay of Salp15 Iric-1 to *B. garinii* VSBP by immunofluorescence

Purified Salp15 Iric-1 was biotinylated by incubating 1 mg/ml Salp15 with 0.25 mg/ml sulfo-NHS-biotin (Pierce) for 45 min at 4°C. Unbound sulfo-NHS-biotin was removed by dialysis against 25 mM lysine in phosphate-buffered saline (PBS) overnight and four times against PBS for 15 min at 4°C. Biotinylated purified Salp15 Iric-1 was incubated with *B. garinii* VSBP spirochetes for 30 min at 33°C. The spirochetes were washed twice with PBS-1% BSA and were resuspended in PBS-1% BSA, air dried on microscope slides overnight, and fixed in 100% methanol. Slides were incubated with bisbenzimide and streptavidine-Cy3 (Sigma) and examined with a fluorescence microscope (Axioscop 2 mot plus; Carl Zeiss).

Detection of terminal C5b-9 complement complexes

*B. garinii* strain VSBP was preincubated with either BSA, Iscap Salp15, or Salp15 Iric-1 for 30 min at 33°C. Then, the spirochetes were incubated in BSK-H medium containing 12.5% NHS for 30 min. The spirochetes were washed twice with PBS-1% BSA. They were resuspended in PBS-1% BSA, air dried on microscope slides overnight, and fixed in 100% methanol. Spirochetes were detected by incubation with human serum containing antibodies against *B. burgdorferi*, and the C5b to C9 (C5b-9; one molecule each of C5b, C6, C7, and C8 and one or more molecules of C9) complement complexes were indicated with monoclonal mouse C5b-9 antibodies (Dako). Slides were washed with PBS-1% BSA and incubated with an anti-human immunoglobulin G-fluorescein isothiocyanate-labeled antibody (BioMerieux) and an anti-mouse Cy3 antibody (Jackson). After slides were washed and were mounted with Mowiol, they were visualized by confocal microscopy using a fluorescence microscope (Axioscop 2 mot plus; Carl Zeiss). At least 100 spirochetes were counted, and the experiment was performed two times.
Statistical analysis

The protection of spirochetes against complement-mediated killing by Salp15 Iric-1 or Iscap Salp15 was compared to the protection by the control protein BSA. The chi-square test was used for the analysis of proportions, where absolute numbers of spirochetes were used in cross tabulations. Crude relative risks for surviving different circumstances were estimated as odds ratios (OR) and presented with both 95% confidence intervals (95% CI) and $P$ values. Tests were performed using SPSS 14 software. Calculated $P$ values of $< 0.05$ were considered significant.

Results

Salp15 protects serum-sensitive *B. burgdorferi* sensu lato isolates against complement-mediated killing

As expected, *B. burgdorferi* sensu lato isolates differed in their sensitivities to NHS. No motile *B. garinii* A87S or *B. garinii* VSBP organisms were seen when incubated for 24 h in 12.5% NHS (Fig. 1). In contrast, other spirochetes required greater amounts of NHS to induce partial killing: *B.
Protection of serum-sensitive Borrelia isolates

B. burgdorferi B31 was killed at 24 h by 50% NHS (Fig. 1) and B. burgdorferi VS215 was killed by 75% NHS at 24 h (data not shown). All these Borrelia isolates survived incubation for 1.5, 4.5, or 24 h with heat-inactivated NHS, demonstrating the importance of complement in serum sensitivity. Since Salp15 has previously been shown to enhance the capacity of spirochetes to survive in naive mice (25), we determined whether Salp15 could alter the serum sensitivity of these Borrelia isolates. Indeed, Salp15 from I. scapularis ticks, Iscap Salp15, altered the serum sensitivity of Borrelia (Fig. 1). The percentage of dead spirochetes significantly decreased when Borrelia isolates were preincubated with Iscap Salp15 (Table 1). The strongest protective effect was seen with B. garinii VSBP. We initially used Salp15 from I. scapularis ticks, but since the serum-sensitive Borrelia are transmitted predominantly by I. ricinus ticks, we determined whether Salp15 from these ticks was as potent as or more potent than Salp15 from I. scapularis for selected Borrelia isolates. We therefore cloned, expressed, and purified Salp15 from I. ricinus, Salp15 Iric-1, as described in Materials and Methods. Salp15 Iric-1 afforded protection against complement-mediated killing (Fig. 1; Table 1). The protective effect of Salp15 Iric-1 for all four Borrelia isolates was also greater.
than that of Iscap Salp15 (Table 1) and the difference in protection of serum-sensitive *B. garinii* spirochetes by Salp15 Iric-1 compared to that by Iscap Salp15 was most apparent (Table 2). A titration of Salp15 showed that both Iscap Salp15 and Salp15 Iric-1 had a dose-dependent protective effect (data not shown).

**Salp15 Iric-1 binds to the surface of *B. garinii* VSBP**

*B. garinii* VSBP was preincubated with biotinylated Salp15 Iric-1 and membrane-bound Salp15 Iric-1 was detected using streptavidin-Cy3 in the immunofluorescence assay. It has previously been shown that Iscap Salp15 binds on the surface of *B. burgdorferi* 297 (25) and we now show that Salp15 Iric-1 also specifically binds to the surface of *B. garinii* VSBP (Fig. 2A) by immunofluorescence.

![Figure 2](image-url)

Figure 2. Salp15 Iric-1 binds to the surfaces of *B. garinii* VSBP spirochetes. Spirochetes were preincubated with biotinylated Salp15 Iric-1 (A) or biotinylated BSA (B). Spirochetes were detected with bisbenzimide (blue) and bound Salp15 or BSA was detected using streptavidin-Cy3 (red). (C) Both Iscap Salp15 and Salp15 Iric-1 bind *B. burgdorferi* N40 (1) and *B. garinii* VSBP (2) in the overlay binding assay. The arrow indicates Salp15 bound to OspC. M, molecular mass. For color figure see page 259.
assay. All spirochetes were found to bind Salp15 Iric-1 on their surfaces. By solid-phase overlay, we demonstrated that both Iscap Salp15 and Salp15 Iric-1 are able to bind to OspC of *B. garinii* VSBP and *B. burgdorferi* N40 (Fig. 2C).

**Surface-bound Salp15 protects spirochetes against complement-mediated killing**

To study whether bound Salp15 protects spirochetes against complement-mediated killing, *B. garinii* was preincubated with Salp15 Iric-1, after which unbound Salp15 was washed away. BSA was used as a control. Spirochetes to which Salp15 had bound were still protected against complement-mediated killing (Fig. 3), and there was a significant difference between the survival of spirochetes after incubation with either BSA or Salp15 and subsequent washing. Notably fewer spirochetes were initially killed in the control group after washing. This could be caused by the loss of less viable spirochetes in the washing procedure. The fact that this difference is not observed among spirochetes preincubated with Salp15 suggests that all spirochetes, independently of initial viability, are potentially protected by Salp15.

**Spirochetes that survive in the presence of Salp15 do not have an increased capacity to bind Salp15**

In the presence of Salp15 Iric-1, 2% of the *B. garinii* VSBP spirochetes survived incubation with 50% NHS for 24 h. The surviving spirochetes were reisolated to determine whether the “survival” phenotype could be enriched via selection. After repeating the selection assay two times, the survival rate of the VSBP subculture was compared to that of the original VSBP isolate after incubation with Salp15 and exposure to 12.5% NHS. The *B. garinii* VSBP subculture was not more protected by Salp15 Iric-1 than the original *B. garinii* VSBP culture (data not shown).

**C5b-9 Deposition is Inhibited by Salp15 Iric-1 and Iscap Salp15**

To examine the influence of Salp15 against complement-mediated killing, we investigated, by use of an immunofluorescence assay, the differences in deposition of C5b-9 terminal complement...
complexes between spirochetes that had been incubated with Salp15 Iric-1, Iscap Salp15, or BSA (control) and then exposed to NHS. When B. garinii VSBP was first incubated with Salp15 Iric-1 or Iscap Salp15, significantly fewer (P < 0.0001) C5b-9 complement complexes were found on the membranes of the spirochetes (Fig. 4A to C). Of the spirochetes that had been incubated with BSA and 12.5% NHS, 90% had C5b-9 complement complexes on their membrane, while 52% and 15% of the spirochetes showed C5b-9 deposition after incubation with Iscap Salp15 and Salp15 Iric-1, respectively (Fig. 4D).

Discussion

In the present study, we investigated the role of the tick salivary protein Salp15 in the protection of B. burgdorferi sensu lato strains against the complement system. Previously, we have shown that there is a great diversity in serum sensitivity among B. burgdorferi sensu lato strains (28). Many B. garinii strains are serum sensitive, while B. burgdorferi and B. afzelii are commonly resistant or intermediately resistant to serum. We now show that both serum-sensitive B. garinii and intermediately resistant B. burgdorferi strains are protected against complement-mediated killing when coincubated with sera and Salp15 from either I. scapularis or I. ricinus (Fig. 1; Table 1). Both Salp15 Iric-1 and Iscap Salp15 appeared to be protective, although Salp15 Iric-1 gave significantly more protection against complement-mediated killing than Iscap Salp15 (Table 2). We were not able to select for a more resistant subpopulation by subculturing spirochetes surviving incubation with 50% NHS and Salp15. When a lysate of the original spirochetes was compared to a lysate of the subcultured spirochetes on a Coomassie-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, no differences in the expression of OspC, which is the natural ligand of Salp15, or of other proteins were found (data not shown).

Serum-resistant strains use CRASPs and Erps to bind plasma proteins factor H and FHL-1, which enables the spirochetes to inactivate the C3 convertase complex (9, 15, 16). Because serum-sensitive
strains do not have their own mechanism to protect themselves against complement-mediated killing, they might benefit from binding proteins from their environment that protect them against this part of the innate immune system, which could explain why serum-sensitive *B. garinii* strains are protected to a great extent in comparison to intermediately resistant *B. burgdorferi* strains (Fig. 1; Table 1). During the blood meal of ticks, Salp15 is secreted by the tick salivary glands, and it was previously shown that Salp15 mRNA levels were 13-fold higher, and Salp15 protein levels were 1.6-fold higher, in salivary glands from engorged ticks infected with *B. burgdorferi* (25). Not only for spirochetes, but also for ticks it is important that the immune system of the host is suppressed. When complement is activated, anaphylatoxins and other proinflammatory mediators are able to trigger degranulation of mast cells and attract phagocytes.
Ticks have a cocktail of salivary proteins which are necessary to take an effective blood meal. It has already been shown that they use Salp15 to inhibit the activation and proliferation of CD4+ T cells by binding to its CD4 receptor (1, 6, 13). In addition, tick salivary proteins have been found to inhibit B cells (8), dendritic cells (4, 11), NK cells (17), neutrophils (20), and macrophages (7). Isac (5, 27) and Salp20 (26) are two salivary proteins that were shown to inhibit the alternative pathway of the complement system. We here describe that a microorganism binding a protein from the vector is protected against killing by the complement system (Fig. 3). Since surface-bound Salp15 was able to protect *Borrelia* even in the absence of free unbound Salp15, it appeared that the protective effect was not caused by the neutralization of C5b-9 formation, at least not completely. When spirochetes were preincubated with Salp15, they were all found to bind Salp15 Iric-1 (Fig. 2) and Iscap Salp15 on their surfaces. For spirochetes, this protection is crucial for survival, since activation of the complement system also initiates the membrane attack pathway, which results in the formation of the MAC consisting of one molecule each of C5b, C6, C7, and C8 and one or more molecules of C9 (Fig. 4). We here demonstrate that Salp15 protected the spirochetes against the formation of MAC complexes. When spirochetes were incubated with Iscap Salp15, deposition of the terminal C5b-9 complement complexes was reduced by 38% compared to what was observed for spirochetes that were incubated with BSA. The effect of Salp15 Iric-1 was even more evident and reduced deposition of C5b-9 by 75% (Fig. 4D). These findings show that Salp15 gives protection not only against bactericidal antibodies but also against the complement system, an important part of the innate immune system. These results could help to explain the higher spirochetal loads in organs of naive mice when inoculated with spirochetes preincubated with Salp15 (25).

In summary, both *I. ricinus* Salp15 and Iscap Salp15 protect *Borrelia* against complement-mediated killing when bound to the membranes of the spirochetes. Protection against the complement system is possibly crucial for the establishment of *B. burgdorferi* infection of the vertebrate host. These findings make it even more interesting to target Salp15 for a vaccine, especially for the prevention of transmission of serum-sensitive *Borrelia* strains.
References


Chapter 6