The Tick Salivary Protein Salp15 Inhibits the Killing of Serum-Sensitive *Borrelia burgdorferi* Sensu Lato Isolates[▽]

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***Borrelia burgdorferi*, the 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 (one molecule each of C5b, C6, C7, and C8 and one or more molecules of 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.**

The Lyme disease agent *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. The ticks have developed several mechanisms to evade both the 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 antihemostatic (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 coreceptor of the T cells (1, 6, 13). Also, Salp15 appeared to enhance 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 found 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 response, the complement system in particular, plays a crucial role in the eradication of invading pathogens. The complement system is important in the initiation of attack 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 are able to activate

complement 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., binding factor H and factor H-like protein-1 (FHL-1) to CRASP-1_{Bb} (15), CRASP-2_{Bb} (9), OspE (10), and/or CRASP-3_{Bb} (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 using *Drosophila* cells expressing *salp15* together with a resistance gene for blastomycin (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, submitted for publication). 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.

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FIG. 1. Spirochetes are protected against complement-mediated killing in the presence of Salp15 Iric-1 or Iscap Salp15. Serum-sensitive *B. garinii* strains VSBP (A) and A87S (B) were incubated with 12.5% NHS, while intermediately resistant *B. burgdorferi* strains VS215 (C) and B31 (D) were incubated with 50% NHS, after they had been preincubated for 30 min with BSA, Iscap Salp15, or Salp15 Iric-1. As a control, spirochetes were also incubated with heat-inactivated NHS. After 1.5 h, 4.5 h, and 24 h of incubation with serum, the percentages of immotile spirochetes were determined. Two hundred spirochetes were counted.

The *Drosophila* cells were subsequently induced with copper sulfate with a final concentration of 500 mM for 4 days and centrifuged at $1,000 \times g$ for 15 min. The supernatant was filtered using a 0.22- μ m 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- μ m 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 (10^7 spirochetes ml⁻¹). Spirochetes (2.5×10^5) were preincubated with bovine serum albumin (BSA), Salp15 Iric-1, or Iscap Salp15 (80 μ g/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 μ g/ml to 160 μ g/ml, were also tested in the same way.

To find out if membrane-bound Salp15 protects the spirochetes against antibody-independent complement-mediated killing, the spirochetes were washed

twice with BSK-H medium ($4,000 \times g$, 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 1×10^8 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 μ g/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,

TABLE 1. Protective effect of Salp15 Iric-1 or Iscap Salp15 compared to that of BSA

Isolate	Preincubation with ^a :	Protection against immobilization after ^b :											
		1.5 h				4.5 h				24 h			
		% Dead ^c	OR ^d	95% CI	P value	% Dead	OR	95% CI	P value	% Dead	OR	95% CI	P value
<i>B. garinii</i> VSBP	BSA	57/43				82/62				93/88			
	Iric-1	13	8.9	5.4–14.6	<0.0001	19	19.4	11.7–32.2	<0.0001	48	14.7	8.0–27.0	<0.0001
		9	7.6	6.0–9.8	<0.0001	22	5.9	4.9–7.2	<0.0001	46	8.2	6.6–10.3	<0.0001
	Iscap	24	4.2	2.7–6.4	<0.0001	59	3.1	2.0–4.9	<0.0001	88	1.9	0.96–3.8	N.S.
<i>B. garinii</i> A87S	BSA	70/71				87/91				97/94			
	Iric-1	34	4.6	3.0–7.1	<0.0001	47	7.6	4.6–12.4	<0.0001	44	35.1	15.7–78.4	<0.0001
		33	5.0	4.2–6.1	<0.0001	64	5.5	4.3–7.0	<0.0001	60	10.9	8.1–14.5	<0.0001
	Iscap	54	2.0	1.4–3.1	0.001	84	1.3	0.73–2.2	N.S.	90	3.1	1.3–7.4	0.01
<i>B. burgdorferi</i> VS215	BSA	75/75				77/80				79/83			
	Iric-1	44	3.8	2.5–5.8	<0.0001	56	2.6	1.7–3.9	<0.0001	55	3.1	2.0–4.7	<0.0001
		53	2.7	2.2–3.3	<0.0001	59	2.7	2.2–3.3	<0.0001	61	3.2	2.6–4.0	<0.0001
	Iscap	59	2.1	1.4–3.3	<0.0001	65	1.8	1.1–2.7	0.01	68	1.8	1.1–2.8	0.01
<i>B. burgdorferi</i> B31	BSA	81/86				82/90				98/89			
	Iric-1	65	2.2	1.4–3.5	<0.0001	75	3.0	1.6–5.4	<0.0001	93	2.9	1.0–8.3	0.03
		74	2.2	1.7–2.7	<0.0001	79	2.3	1.8–3.0	<0.0001	77	2.6	2.0–3.3	<0.0001
	Iscap	71	1.7	1.1–2.7	0.03	71	1.5	0.76–2.8	N.S.	93	2.9	1.0–8.3	0.03
		79	1.6	1.3–2.1	<0.0001	83	1.8	1.4–2.4	<0.0001	78	2.4	1.9–3.1	<0.0001

^a Iric-1 and Iscap indicate Salp15 Iric-1 and Iscap Salp15, respectively. Spirochetes were preincubated with Salp15 Iric-1 or Iscap Salp15 for 30 min before incubation with NHS.

^b Spirochetes were incubated for 1.5, 4.5, or 24 h with 12.5% NHS in the case of *B. garinii* VSBP and *B. garinii* A87S or 50% NHS in the case of *B. burgdorferi* B31 and VS215.

^c Percentages of dead spirochetes. One hundred spirochetes were counted and the experiment was performed two times. Lightface values show the percentages of dead spirochetes treated with Salp15 Iric-1, while boldface values indicate the percentages of dead spirochetes treated with Iscap Salp15.

^d The protective effect of Salp15 compared to BSA was calculated using the chi-square test. Lightface values represent experiments with 200 spirochetes, while boldface values indicate experiments with 1,000 spirochetes.

and fixed in 100% methanol. Slides were incubated with bisbenzimidazole 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. 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

TABLE 2. Protective effect of Salp15 Iric-1 compared to that of Iscap Salp15

Isolate	Protection against immobilization after ^a :											
	1.5 h				4.5 h				24 h			
	% Dead ^b	OR ^c	95% CI	P value	% Dead	OR	95% CI	P value	% Dead	OR	95% CI	P value
<i>B. garinii</i> VSBP	9/25	3.3	2.6–4.3	<0.0001	22/49	3.5	2.9–4.2	<0.0001	46/83	5.6	4.6–6.9	<0.0001
<i>B. garinii</i> A87S	33/51	2.1	1.8–2.5	<0.0001	64/89	4.4	3.5–5.6	<0.0001	60/89	5.2	4.2–6.6	<0.0001

^a Spirochetes were incubated for 1.5, 4.5, or 24 h with 12.5% NHS.

^b Percentages of dead spirochetes. One hundred spirochetes were counted and the experiment was performed two times. Lightface values show the percentages of dead spirochetes treated with Salp15 Iric-1, while boldface values indicate the percentages of dead spirochetes treated with Iscap Salp15.

^c The protective effect of Salp15 Iric-1 versus Iscap Salp15 against complement-mediated killing was calculated using the chi-square test.

FIG. 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 bisbenzimidazole (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.

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 *B. burgdorferi* 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 had previously cloned Salp15 from *I. ricinus* ticks, but since the serum-sensitive *Borrelia* are transmitted predominantly by *I. ricinus* ticks, we wanted to determine 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 assay. By solid-phase overlay, we demon-

strated that both Iscap Salp15 and Salp15 Iric-1 are able to bind to OspC of *B. garinii* VSBP and *B. burgdorferi* N40 (Fig. 2C). All spirochetes were found to bind Salp15 Iric-1 on their surfaces.

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 been bound were still protected

FIG. 3. Membrane-bound Salp15 Iric-1 protects *B. garinii* VSBP against complement-mediated killing. Spirochetes were preincubated with BSA or Salp15 for 60 min. Subsequently, BSA or Salp15 was removed by washing as indicated in Materials and Methods, and spirochetes were exposed to 12.5% NHS. Percentages of motile spirochetes were determined for samples and for controls in which Salp15 was still present. Standard deviations, based on duplicate countings of two independent experiments, are included. The asterisks indicate a statistically significant difference between the groups ($P < 0.0001$).

FIG. 4. Inhibition of C5b-9 deposition by Salp15. *B. garinii* VSBP was preincubated with BSA (A), Iscap Salp15 (B), or Salp15 Iric-1 (C) before being subjected to 12.5% NHS. Spirochetes were double labeled with antibodies specific for *Borrelia* spirochetes (green) and specific for C5b-9 complement complexes (red). (D) Deposition of C5b-9 complexes was strongly inhibited in the presence of Iscap Salp15 and even more by Salp15 Iric-1. Bars indicate standard deviations, based on duplicate countings of two independent experiments. One hundred spirochetes were counted, and the experiment was performed two times. The asterisk indicates a statistically significant difference

against complement-mediated killing (Fig. 3), and there was a significant difference between the survivals 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.

C5b-9 deposition is inhibited by Salp15 Iric-1 or 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 found that both serum-sensitive *B. garinii* and intermediately resistant *B. burgdorferi* strains were 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

($P < 0.0001$) between the BSA- and Salp15 Iric-1-treated groups, the double asterisk shows a significant difference ($P < 0.0001$) between the BSA- and Iscap Salp15-treated groups, and the triple asterisk indicates a significant difference ($P < 0.0001$) between the Iscap Salp15- and Salp15 Iric-1-treated groups.

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 explains why serum-sensitive *B. garinii* strains are protected to a great extent in comparison to immediately resistant *B. burgdorferi* strains (Fig. 1; Table 1).

During the blood meal of ticks, Salp15 is secreted into the tick salivary glands, and it was shown that Salp15 mRNA levels were 13-fold higher and Salp15 protein levels in the salivary glands were 1.6-fold higher in engorged ticks when they were infected with *B. burgdorferi* (25). Not only for spirochetes, but also for ticks it is extremely 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. Additionally, this may explain why the ticks infected with spirochetes upregulate *salp15*.

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. This is the first description of a microorganism binding from the vector a protein that protects the pathogen against 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 MAC consisting of one molecule each of C5b, C6, C7, and C8 and one or more molecules of C9 (Fig. 4). We also demonstrated that Salp15 protects the spirochetes against the formation of the MAC complex. When spirochetes were incubated with Iscap, Salp15 deposition of the terminal C5b-9 complement complexes was reduced by 38% compared to what was seen 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, a very important part of the innate immune system. These

results can also explain why higher spirochetal loads in organs of naive mice were found when the inoculated spirochetes were preincubated with Salp15 (25).

In summary, both *I. ricinus* Salp15 and Iscap Salp15 are able to give protection against complement-mediated killing when bound on the membranes of *Borrelia* spirochetes. The spirochetal protecting effect against the complement system is possibly crucial in the initial 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 strains.

REFERENCES

- Anguita, J., N. Ramamoorthi, J. W. Hovius, S. Das, V. Thomas, R. Persinski, D. Conze, P. W. Askenase, M. Rincon, F. S. Kantor, and E. Fikrig. 2002. Salp15, an Ixodes scapularis salivary protein, inhibits CD4(+) T cell activation. *Immunity* 16:849–859.
- Binnington, K. C., and D. H. Kemp. 1980. Role of tick salivary glands in feeding and disease transmission. *Adv. Parasitol.* 18:315–339.
- Breitner-Ruddock, S., R. Wurzner, J. Schulze, and V. Brade. 1997. Heterogeneity in the complement-dependent bacteriolysis within the species of *Borrelia burgdorferi*. *Med. Microbiol. Immunol.* 185:253–260.
- Cavassani, K. A., J. C. Aliberti, A. R. Dias, J. S. Silva, and B. R. Ferreira. 2005. Tick saliva inhibits differentiation, maturation and function of murine bone-marrow-derived dendritic cells. *Immunology* 114:235–245.
- Daix, V., H. Schroeder, N. Praet, J. P. Georgin, I. Chiappino, L. Gillet, K. de Fays, Y. Decrem, G. Lebouille, E. Godfroid, A. Bollen, P. P. Pastoret, L. Gern, P. M. Sharp, and A. Vanderplassechen. 2007. Ixodes ticks belonging to the Ixodes ricinus complex encode a family of anticomplement proteins. *Insect Mol. Biol.* 16:155–166.
- Garg, R., I. J. Juncadella, N. Ramamoorthi, Ashish, S. K. Ananthanarayanan, V. Thomas, M. Rincon, J. K. Krueger, E. Fikrig, C. M. Yengo, and J. Anguita. 2006. Cutting edge: CD4 is the receptor for the tick saliva immunosuppressor, Salp15. *J. Immunol.* 177:6579–6583.
- Gwakisa, P., K. Yoshihara, T. T. Long, H. Gotoh, F. Amano, and E. Momotani. 2001. Salivary gland extract of *Rhipicephalus appendiculatus* ticks inhibits in vitro transcription and secretion of cytokines and production of nitric oxide by LPS-stimulated JA-4 cells. *Vet. Parasitol.* 99:53–61.
- Hannier, S., J. Liversidge, J. M. Sternberg, and A. S. Bowman. 2004. Characterization of the B-cell inhibitory protein factor in *Ixodes ricinus* tick saliva: a potential role in enhanced *Borrelia burgdorferi* transmission. *Immunology* 113:401–408.
- Hartmann, K., C. Corvey, C. Skerka, M. Kirschfink, M. Karas, V. Brade, J. C. Miller, B. Stevenson, R. Wallich, P. F. Zipfel, and P. Kraiczky. 2006. Functional characterization of BbCRASP-2, a distinct outer membrane protein of *Borrelia burgdorferi* that binds host complement regulators factor H and FHL-1. *Mol. Microbiol.* 61:1220–1236.
- Hellwage, J., T. Meri, T. Heikkilä, A. Alitalo, J. Panelius, P. Lahdenne, I. J. Seppala, and S. Meri. 2001. The complement regulator factor H binds to the surface protein OspE of *Borrelia burgdorferi*. *J. Biol. Chem.* 276:8427–8435.
- Hovius, J. W., M. A. W. P. de Jong, J. den Dunnen, M. Litjens, E. Fikrig, T. van der Poll, S. I. Gringhuis, and T. B. H. Geijtenbeek. 2008. Salp15 binding to DC-SIGN inhibits cytokine expression by impairing both nucleosome remodeling and mRNA stabilization. *PLoS Pathog.* 4:e31. doi:10.1371/journal.ppat.0040031.
- Hovius, J. W., N. Ramamoorthi, C. Van't Veer, K. A. de Groot, A. M. Nijhof, F. Jongejan, A. P. van Dam, and E. Fikrig. 2007. Identification of Salp15 homologues in *Ixodes ricinus* ticks. *Vector Borne Zoonotic Dis.* 7:296–303.
- Juncadella, I. J., R. Garg, S. K. Ananthanarayanan, C. M. Yengo, and J. Anguita. 2007. T-cell signaling pathways inhibited by the tick saliva immunosuppressor, Salp15. *FEMS Immunol. Med. Microbiol.* 49:433–438.
- Kotsyfakis, M., A. Sa-Nunes, I. M. Francischetti, T. N. Mather, J. F. Andersen, and J. M. Ribeiro. 2006. Antiinflammatory and immunosuppressive activity of sialostatin L, a salivary cystatin from the tick *Ixodes scapularis*. *J. Biol. Chem.* 281:26298–26307.
- Kraiczky, P., J. Hellwage, C. Skerka, H. Becker, M. Kirschfink, M. M. Simon, V. Brade, P. F. Zipfel, and R. Wallich. 2004. Complement resistance of *Borrelia burgdorferi* correlates with the expression of BbCRASP-1, a novel linear plasmid-encoded surface protein that interacts with human factor H and FHL-1 and is unrelated to Erp proteins. *J. Biol. Chem.* 279:2421–2429.
- Kraiczky, P., J. Hellwage, C. Skerka, M. Kirschfink, V. Brade, P. F. Zipfel, and R. Wallich. 2003. Immune evasion of *Borrelia burgdorferi*: mapping of a complement-inhibitor factor H-binding site of BbCRASP-3, a novel member of the Erp protein family. *Eur. J. Immunol.* 33:697–707.
- Kubes, M., P. Kocakova, M. Slovák, M. Slavikova, N. Fuchsberger, and P. A. Nuttall. 2002. Heterogeneity in the effect of different ixodid tick species on human natural killer cell activity. *Parasite Immunol.* 24:23–28.
- Kyckova, K., and J. Kopecky. 2006. Effect of tick saliva on mechanisms of

- innate immune response against *Borrelia afzelii*. *J. Med. Entomol.* **43**:1208–1214.
19. Lawrie, C. H., S. E. Randolph, and P. A. Nuttall. 1999. Ixodes ticks: serum species sensitivity of anticomplement activity. *Exp. Parasitol.* **93**:207–214.
 20. Montgomery, R. R., D. Lusitani, C. A. De Boisfleury, and S. E. Malawista. 2004. Tick saliva reduces adherence and area of human neutrophils. *Infect. Immun.* **72**:2989–2994.
 21. Narasimhan, S., R. A. Koski, B. Beaulieu, J. F. Anderson, N. Ramamoorthi, F. Kantor, M. Cappello, and E. Fikrig. 2002. A novel family of anticoagulants from the saliva of *Ixodes scapularis*. *Insect Mol. Biol.* **11**:641–650.
 22. Nazareth, R. A., L. S. Tomaz, S. Ortiz-Costa, G. C. Atella, J. M. Ribeiro, I. M. Francischetti, and R. Q. Monteiro. 2006. Antithrombotic properties of Ixolaris, a potent inhibitor of the extrinsic pathway of the coagulation cascade. *Thromb. Haemost.* **96**:7–13.
 23. Patarakul, K., M. F. Cole, and C. A. Hughes. 1999. Complement resistance in *Borrelia burgdorferi* strain 297: outer membrane proteins prevent MAC formation at lysis susceptible sites. *Microb. Pathog.* **27**:25–41.
 24. Pausa, M., V. Pellis, M. Cinco, P. G. Giulianini, G. Presani, S. Perticarari, R. Murgia, and F. Tedesco. 2003. Serum-resistant strains of *Borrelia burgdorferi* evade complement-mediated killing by expressing a CD59-like complement inhibitory molecule. *J. Immunol.* **170**:3214–3222.
 25. Ramamoorthi, N., S. Narasimhan, U. Pal, F. Bao, X. F. Yang, D. Fish, J. Anguita, M. V. Norgard, F. S. Kantor, J. F. Anderson, R. A. Koski, and E. Fikrig. 2005. The Lyme disease agent exploits a tick protein to infect the mammalian host. *Nature* **436**:573–577.
 26. Tyson, K., C. Elkins, H. Patterson, E. Fikrig, and A. de Silva. 2007. Biochemical and functional characterization of Salp20, an *Ixodes scapularis* tick salivary protein that inhibits the complement pathway. *Insect Mol. Biol.* **16**:469–479.
 27. Valenzuela, J. G., R. Charlab, T. N. Mather, and J. M. Ribeiro. 2000. Purification, cloning, and expression of a novel salivary anticomplement protein from the tick, *Ixodes scapularis*. *J. Biol. Chem.* **275**:18717–18723.
 28. van Dam, A. P., A. Oei, R. Jaspars, C. Fijen, B. Wilske, L. Spanjaard, and J. Dankert. 1997. Complement-mediated serum sensitivity among spirochetes that cause Lyme disease. *Infect. Immun.* **65**:1228–1236.

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