

The Lyme disease agent exploits a tick protein to infect the mammalian host

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The Lyme disease agent, *Borrelia burgdorferi*, is maintained in a tick–mouse cycle^{1,2}. Here we show that *B. burgdorferi* usurps a tick salivary protein, Salp15 (ref. 3), to facilitate the infection of mice. The level of *salp15* expression was selectively enhanced by the presence of *B. burgdorferi* in *Ixodes scapularis*, first indicating that spirochaetes might use Salp15 during transmission. Salp15 was then shown to adhere to the spirochaete, both *in vitro* and *in vivo*, and specifically interacted with *B. burgdorferi* outer surface protein C. The binding of Salp15 protected *B. burgdorferi* from antibody-mediated killing *in vitro* and provided spirochaetes with a marked advantage when they were inoculated into naive mice or animals previously infected with *B. burgdorferi*. Moreover, RNA interference-mediated repression of *salp15* in *I. scapularis* drastically reduced the capacity of tick-borne spirochaetes to infect mice. These results show the capacity of a pathogen to use a secreted arthropod protein to help it colonize the mammalian host.

Lyme borreliosis serves as a model to examine how microbe–vector interactions influence pathogen transmission to the mammalian host. *B. burgdorferi*, the spirochetal agent of Lyme disease, is primarily maintained in the USA in *I. scapularis* ticks and *Peromyscus leucopus* mice. Spirochaetes preferentially express specific genes to survive in a complex enzootic cycle⁴. For example, *B. burgdorferi* cells entering *I. scapularis* express outer surface protein (Osp)A to colonize the vector⁵. When the arthropod engorges on a host, the spirochaetes then downregulate OspA and upregulate OspC, a lipoprotein that facilitates the migration of *B. burgdorferi* from the

I. scapularis gut to the tick salivary glands and can independently participate in the establishment of vertebrate infection^{6,7}. While *B. burgdorferi* are being transmitted during tick feeding, the arthropod is also secreting saliva to aid in engorgement⁸. *I. scapularis* saliva possesses antigens with immunosuppressive, anticomplement and antihemostatic activity, among other functions, which enable the vector to take an effective blood meal^{9,10}. We now explore the hypothesis that *B. burgdorferi* in transit through the tick might use components of *I. scapularis* saliva to enhance spirochaete transmission to, and survival within, the vertebrate host.

To determine first whether *B. burgdorferi* influenced the expression of tick genes, we examined the profile of the genes encoding 14 antigenic *I. scapularis* salivary proteins that elicit strong humoral responses in the host upon tick feeding¹¹, in uninfected and *B. burgdorferi*-infected ticks. We found that the expression of one gene, *salp15*, which encodes an *I. scapularis* protein known to inhibit T-cell activation³, was selectively increased in *B. burgdorferi*-infected tick salivary glands during engorgement (Fig. 1a). As an example of one control, the expression of the gene encoding another salivary antigen, *salp25D*, remained the same regardless of infection. Quantitative polymerase chain reaction (PCR) further showed that *salp15* mRNA levels were 13-fold higher ($P < 0.001$) in *B. burgdorferi*-infected engorged ticks than in engorged *I. scapularis* without *B. burgdorferi* (Fig. 1b). In contrast, the amount of *salp25D* mRNA was similar in both groups of ticks (Fig. 1b). The enhancement in *salp15* expression was specific to *B. burgdorferi*, because the levels of *salp15* mRNA in the salivary glands of engorged ticks infected with

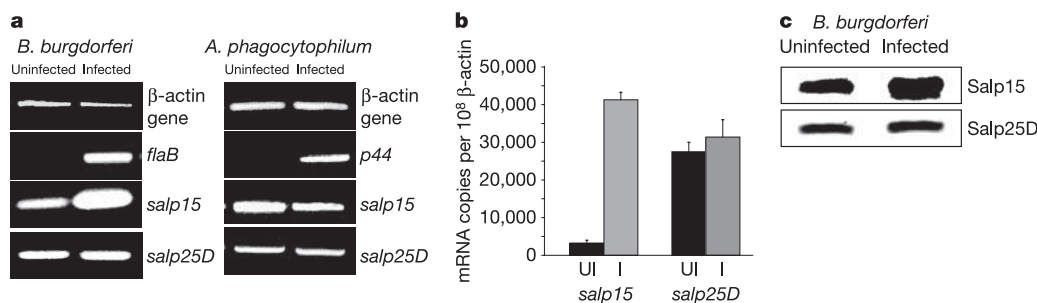


Figure 1 | Salp15 levels are specifically enhanced in *Borrelia burgdorferi*-infected tick salivary glands. **a**, RT–PCR profile of fed *Ixodes scapularis* salivary glands that were uninfected or infected with either *B. burgdorferi* or *Anaplasma phagocytophilum*. Expression of the β -actin gene from *I. scapularis* was used as a control. *flaB* and *p44* expression are indicative of *B. burgdorferi* and *A. phagocytophilum* infection, respectively. **b**, *salp15* was

upregulated in *B. burgdorferi*-infected *I. scapularis* salivary glands. The difference between *salp15* mRNA levels in infected and uninfected nymphs was significant, in contrast to *salp25D* expression (Student's *t*-test). Results are means + s.e.m. from three quantitative PCR experiments. **c**, Salp15 protein levels were 1.6-fold higher in *B. burgdorferi*-infected salivary glands, as quantified by ImageJ (NIH). Salp25D served as a control.

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Anaplasma phagocytophilum, another pathogen borne by *I. scapularis*, remained unchanged (Fig. 1a). Salp15 protein levels were 1.6-fold higher in *B. burgdorferi*-infected salivary glands than in uninfected glands, as demonstrated by immunoblotting (Fig. 1c). The selective upregulation of a tick salivary antigen in the presence of *B. burgdorferi* raises the possibility that Salp15 might be used by the pathogen, either during its interim stay in the arthropod salivary gland or during its transit into the mammalian host.

To assess whether Salp15 interacts with a *B. burgdorferi* antigen, a gel overlay assay, using recombinant Salp15, was performed. Salp15 bound a 22-kDa *B. burgdorferi* antigen that was identified as OspC (Fig. 2a) when subjected to matrix-assisted laser desorption/ionization mass spectrometry peptide analysis (Supplementary Table S1). Consistent with this was our observation that Salp15 did not bind to lysates of OspC-deficient *B. burgdorferi* but did adhere to OspC-deficient *B. burgdorferi* that were genetically complemented to produce OspC (Fig. 2b). In addition to binding the spirochaete lysates, we also observed that Salp15 interacted with intact wild-type *B. burgdorferi*, but not with OspC-deficient *B. burgdorferi*, *in vitro* (Fig. 2c). These observations further confirmed that the Salp15–OspC interaction was specific. Moreover, *B. burgdorferi* in infected tick salivary glands were copiously covered with native Salp15, as detected with an antibody directed against recombinant Salp15 (Fig. 2d). As a control, an antibody against another tick salivary protein, Salp25D, failed to bind to *B. burgdorferi* in the salivary gland. Uninfected salivary glands stained diffusely for both proteins (data not shown). Salp15 therefore directly associates with *B. burgdorferi* within the vector.

The enhanced expression of Salp15 in the presence of *B. burgdorferi* within ticks, and the specific adherence of Salp15 to OspC on the surface of *B. burgdorferi*, suggest a critical role for Salp15 in establishing spirochaete infection. To determine whether Salp15 influenced the ability of *B. burgdorferi* to colonize the mammalian host, spirochaetes were preincubated with Salp15 and injected into naive C3H mice. At 25 days the spirochaete load in animals that received *B. burgdorferi* and Salp15 was markedly elevated in the joints (ninefold; $P < 0.001$), skin (fivefold; $P < 0.001$) and bladder (25-fold; $P < 0.001$) as measured by quantitative PCR (Fig. 3a). The levels were higher than in mice that received *B. burgdorferi* alone (Fig. 3a) or in mice in which Salp15 was injected at a distal site from the *B. burgdorferi* inoculum (Supplementary Fig. S1). The antibody titres to *B. burgdorferi* was similar in all groups of mice. Spirochaete levels were also increased to a similar degree at earlier time points (8 and 15 days) in mice that received *B. burgdorferi* preincubated with Salp15 (data not shown). These results are consistent with previous reports that inoculation of *B. burgdorferi* together with tick salivary gland lysates enhanced the spirochaete load in mice¹².

Innate and adaptive immune responses contribute to controlling the levels of *B. burgdorferi* during infection^{13,14}. In particular, humoral immunity has repeatedly been shown to destroy spirochaetes, both *in vitro* and *in vivo*^{15–17}. Antibody-mediated killing assays were therefore performed to determine whether Salp15 could protect spirochaetes from the borreliacidal effects of *B. burgdorferi* antisera. Cultured wild-type *B. burgdorferi* were killed by a monoclonal antibody against OspA, sera from *B. burgdorferi*-infected mice (data not shown) or rabbit *B. burgdorferi* antisera within 18 h, whereas spirochaetes preincubated with Salp15 were significantly protected (Fig. 3b, c). This effect was not noted with OspC-deficient spirochaetes but was evident with OspC-deficient *B. burgdorferi* that were genetically complemented to produce OspC (data not shown). Microscopic observation revealed an 8.5-fold higher level of viable spirochaetes. The *B. burgdorferi* remained viable until 24 h after exposure to the antisera (Fig. 3b, c). Beyond this time, the Salp15-treated spirochaetes began dying, perhaps because dividing spirochaetes lacking the protective interaction with Salp15 might have been targeted by the borreliacidal antibodies.

In areas where Lyme disease is endemic, mice are repeatedly

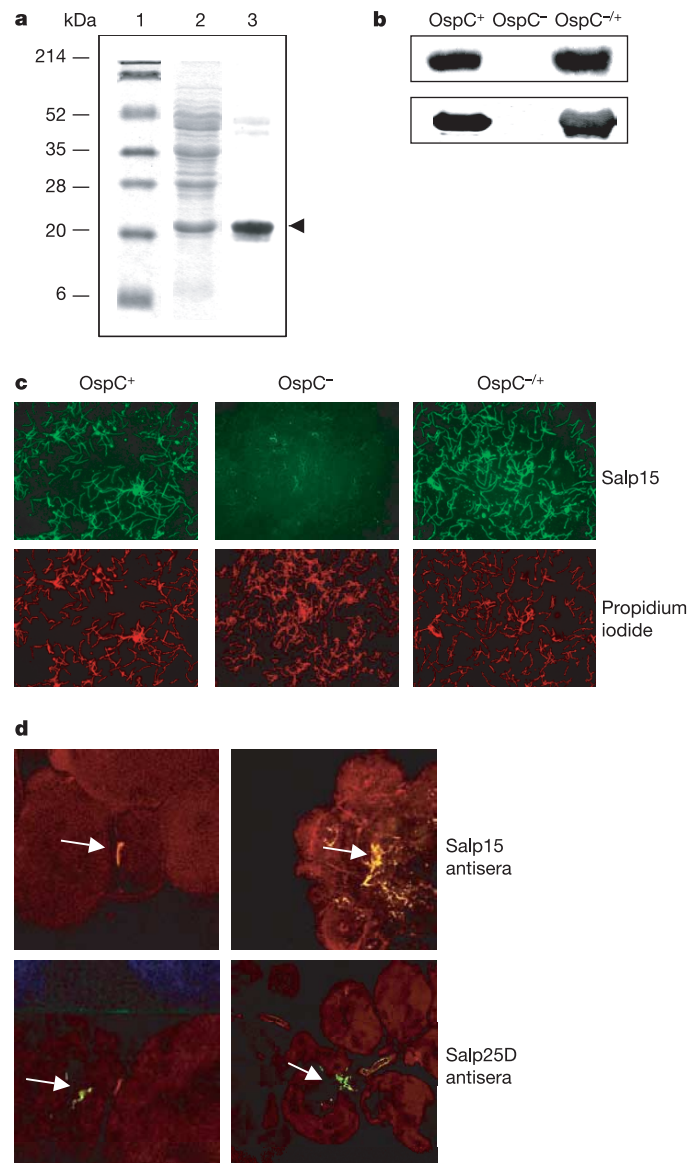


Figure 2 | Salp15 interacts with outer surface protein (OspC) of *Borrelia burgdorferi*. **a**, Salp15 bound specifically to OspC. Lanes 1 and 2, Ponceau S stain of molecular mass markers (lane 1) and *B. burgdorferi* lysate (lane 2); lane 3, Salp15 overlay. The protein band bound by Salp15 (marked by an arrowhead) was identified as OspC. **b**, Salp15 binding to OspC was confirmed by using wild-type (OspC⁺), OspC-deficient (OspC⁻) and OspC-complemented (OspC^{-/+}) *B. burgdorferi*. Bacterial lysates of the isolates were probed with anti-Salp15 (top) and anti-OspC (bottom) antibody. **c**, Salp15 binds to the surface of intact *B. burgdorferi*. Unfixed wild-type (OspC⁺), OspC-deficient (OspC⁻) and OspC-complemented (OspC^{-/+}) *B. burgdorferi* were probed with FITC-conjugated Salp15 (green) and propidium iodide (red). Original magnification $\times 40$. **d**, Salp15 binds *B. burgdorferi* within the tick salivary gland. Top: salivary glands from *B. burgdorferi*-infected nymphs were probed with Salp15 antibody (red). The spirochaetes (as indicated by arrows) were stained with an FITC-labelled anti-*B. burgdorferi* antibody (green). Bottom: anti-Salp25D served as a control. Co-localization (yellow) was observed with Salp15 antisera, in contrast to Salp25D. Left, salivary gland with a single spirochaete; right, a cluster of *B. burgdorferi* is harboured. Images are representative of ten independent experiments. Original magnification $\times 40$.

exposed to ticks with *B. burgdorferi*, and often have evidence of a humoral response to the spirochaete¹⁸. Therefore, in nature, *B. burgdorferi* transmitted by tick bites are frequently exposed to *B. burgdorferi*-specific antibodies¹⁹. This is the environment—during the natural infection of mice—in which the influence of Salp15 is

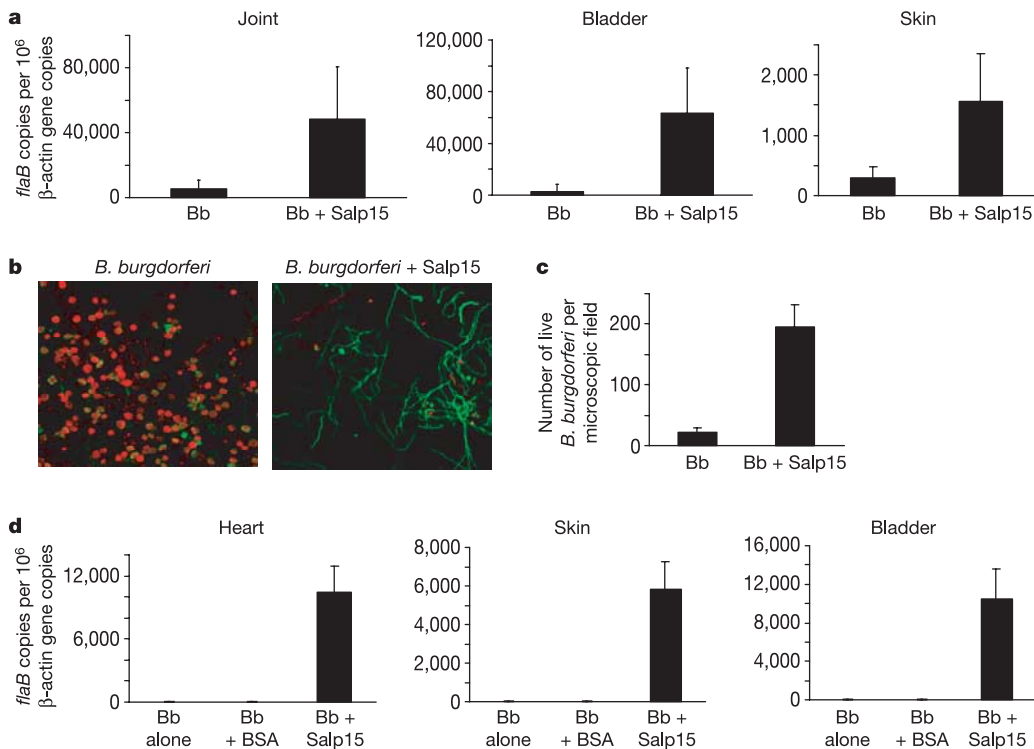


Figure 3 | Salp15 markedly enhances the *Borrelia burgdorferi* load in the murine host. **a**, Naive mice were inoculated with *B. burgdorferi* in the presence (Bb + Salp15) and absence (Bb) of recombinant Salp15. *B. burgdorferi* *flaB* was measured in the different tissues. Results are means + s.e.m. from three independent experiments. **b**, Salp15 protects *B. burgdorferi* from antibody-mediated destruction *in vitro*. *B. burgdorferi* antiserum was incubated with wild-type spirochaetes for 18 h. Live bacteria stained green (Syto 9 stain); dead bacteria stained red (propidium iodide). Left, untreated *B. burgdorferi*; right, *B. burgdorferi* preincubated with Salp15. Original magnification $\times 63$. **c**, Quantitative assessment of the data shown in **b**. After exposure to antibody, *B. burgdorferi* preincubated with

Salp15 were 8.5-fold more viable than the untreated *B. burgdorferi*. Numbers are averages of 20 random microscopic fields. Results are means + s.e.m. from one representative experiment. **d**, Protection from *in vivo* killing of *B. burgdorferi* by Salp15 in immune mice. Salp15 markedly enhanced the *B. burgdorferi* load in a previously infected murine host. Mice were treated as described in Methods. The different groups of mice received phosphate-buffered saline (Bb), bovine serum albumin (Bb + BSA) as control, or Salp15 (Bb + Salp15). *B. burgdorferi* *flaB* levels were quantified from the different tissues. Results are means + s.e.m. from three quantitative PCR experiments.

likely to be most important. In laboratory experiments, tick-borne spirochaetes are more resistant than cultured spirochaetes to being killed *in vivo* by immune sera²⁰. It was therefore important to examine whether the interaction between Salp15 and *B. burgdorferi* could enable spirochaetes to colonize mice that had previously developed an immune response to *B. burgdorferi*. To mimic this situation, C3H mice were infected with *B. burgdorferi* for 14 days, a period during which a protective anti-*B. burgdorferi* humoral response to infection has been noted²⁰, and the mice were then treated with ceftriaxone, an antibiotic that eradicates spirochaete infection²¹. As expected, these animals were resistant to infection with *in vitro* cultured *B. burgdorferi* administered alone or in the presence of bovine serum albumin (Fig. 3d). In contrast, these mice were fully susceptible to infection with spirochaetes that had been preincubated with Salp15 (Fig. 3d). Quantitative PCR performed 25 days after infection detected *B. burgdorferi* only in mice that received spirochaetes with Salp15, and not in animals that received *B. burgdorferi* alone. Moreover, the spirochaete burdens were comparable to those in the non-immune mice infected with *B. burgdorferi* in the presence of Salp15. Taken together, these data indicate that Salp15 allows *B. burgdorferi* to successfully colonize mice that had been previously exposed to spirochaetes and also indicate that Salp15 might be important in spirochaete colonization and dissemination.

To further examine the role of Salp15 in *B. burgdorferi* survival *in vivo*, *salp15*-deficient *I. scapularis* nymphs were generated by RNA interference (RNAi). The successful downregulation of *salp15* expression in tick salivary glands was determined by PCR with

reverse transcription (RT-PCR) (Fig. 4a). The unchanged levels of *salp25D* and tick β -actin gene confirmed the specificity of RNAi. Buffer-injected (mock) and *salp15* double-stranded RNA (dsRNA)-injected *B. burgdorferi*-infected nymphs were then fed on naive laboratory mice and on mice that had previously developed an immune response to *B. burgdorferi*. Quantitative PCR revealed a significant decrease in spirochaete levels in the skin of mice that were fed on by the *salp15*-deficient ticks in comparison with mock-injected ticks, both in naive mice (Fig. 4b; $P < 0.01$) and in mice that had an immune response (Fig. 4c; $P < 0.001$). In contrast, spirochaete levels in the salivary glands of engorged *salp15*-deficient and control tick salivary glands ($2.9 \pm 1.3 \times 10^2$ and $(3.1 \pm 2.1) \times 10^2$ *flaB* copies per 10^6 β -actin gene copies, respectively, ($P > 0.5$) were similar. Spirochaete numbers in the host were unaffected for *salp15*-deficient nymphs (data not shown). Studies were also performed with *P. leucopus*, a natural reservoir of *B. burgdorferi*, and similar results were obtained when *salp15* dsRNA injected *B. burgdorferi*-infected nymphs were fed on naive *P. leucopus* mice and on mice that had an immune response to *B. burgdorferi* (Supplementary Fig. S2a, b). The *salp15*-deficient ticks showed no alterations in engorgement, ruling out the possibility of reduced transmission as a result of decreased feeding. The lack of complete abrogation of pathogen transmission in *salp15*-deficient *I. scapularis* might be due to the inability of RNAi to suppress gene expression totally or due to the spirochaetes' ability to compensate for the absence of *salp15*. These *in vivo* data show conclusively that Salp15 facilitates tick-borne *B. burgdorferi* infection in the host.

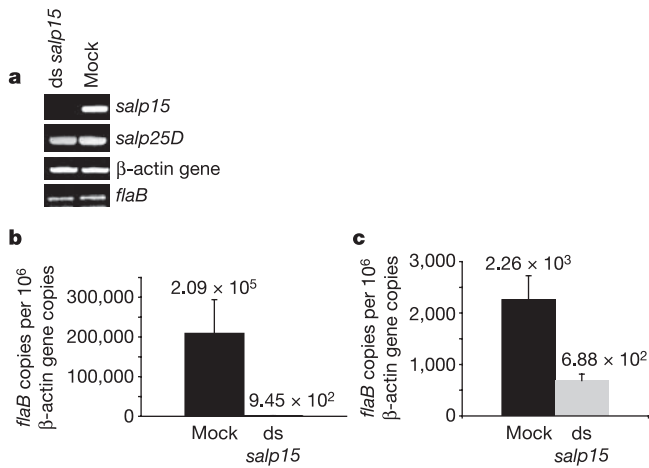


Figure 4 | Gene silencing of *salp15* expression by RNA interference.

a, *Borrelia burgdorferi*-infected nymphal ticks were microinjected with *salp15* dsRNA (ds *salp15*) or buffer alone (mock), and fed on naive mice. Levels of *salp15*, *salp25D* and β -actin gene were assessed in the salivary gland by RT-PCR. Levels of *flaB* were also measured. Data are representative of three independent experiments. **b, c**, *salp15* dsRNA reduced the transmission of *B. burgdorferi* to the host. Nymphal ticks were injected with *salp15* dsRNA (ds *salp15*) or buffer alone (mock), fed on naive mice or on mice previously infected with *B. burgdorferi* and then treated as described in Methods. Quantification of *flaB* revealed lower levels of spirochaete in the skin of mice that were fed upon by *salp15*-deficient ticks in both the naive mice (**b**) and the preimmune mice (**c**) in comparison with mock-injected ticks (Student's *t*-test). Results are means \pm s.e.m. and data are representative of three independent experiments.

Transmission of an arthropod-borne pathogen occurs at the complex interface of microbe, vector and vertebrate host. Although host–pathogen, vector–pathogen and vector–host interactions have been delineated^{22,23}, we now describe a triangular relationship in which an infectious agent exploits an arthropod protein to facilitate infection of the mammalian host. The use of a specific *I. scapularis* salivary protein by *B. burgdorferi* to enhance infection in mice serves as a model for the other arthropod-borne infections. In particular, the presence of *B. burgdorferi* within the vector induces the expression of Salp15 in ticks, and this pathogen–vector interaction is needed for the establishment of *B. burgdorferi* infection in mice that have previously been exposed to the spirochaetes, the most common reservoir host environment that this microbe encounters naturally. Although these present data show that Salp15 is used by *B. burgdorferi* to augment infection, the increased Salp15 levels in ticks during spirochaete infection could also be advantageous to *I. scapularis*. Perhaps *B. burgdorferi*-induced enhancement of Salp15, with its immunosuppressive properties³, might enable ticks to more effectively engorge and/or avoid rejection by the host²⁴, providing a preferential survival advantage for both *I. scapularis* and *B. burgdorferi*. As microbes and vectors have evolved together over millions of years, the most successful relationship between them will be mutual, rather than parasitic or commensal, as the interaction between *B. burgdorferi* and *I. scapularis* may well be. The vector and pathogen factors that influence successful microbial infection of the mammalian host might also serve as targets for vaccines and therapeutics to combat arthropod-borne diseases.

METHODS

Spirochaetes. A low-passage clonal isolate of *Borrelia burgdorferi* strain N40 that is infectious in mice²⁵ was used throughout the study. Clonal isolates of OspC-deficient and OspC-complemented and wild-type *B. burgdorferi* 297 (ref. 6) were used for immunofluorescence and *in vitro* binding assays.

Mice. C3H/HeJ (C3H) mice were purchased from the Jackson Laboratory. *Peromyscus leucopus* were obtained from a colony at Yale University, maintained by D. Fish.

Ticks. *Ixodes scapularis* nymphs were obtained from The Connecticut Agricultural Experiment Station. Laboratory-infected *I. scapularis* nymphs were obtained as described previously²⁶. The uninfected and infected nymphs were fed on pathogen-free C3H mice and were collected after 66 h of feeding for the dissection of salivary glands.

RNA extractions from tick salivary glands. RNA was extracted from the following tick pools: uninfected and infected nymphal tick salivary glands, in accordance with the kit manufacturer's directions (AquaPure RNA isolation kit; Bio-Rad). The RNA samples purified from these groups were used in the qualitative RT-PCR and quantitative PCR assays.

RT-PCR and quantitative PCR. The primer pairs for each assayed gene are listed in Supplementary Table S2. Tick salivary gland complementary DNA was made with the iScript cDNA synthesis kit (Bio-Rad) in accordance with the manufacturer's protocol. Quantitative PCR was performed in accordance with the manufacturer's protocol with a Bio-Rad i-Cycler. The probes used contained a 5' reporter, 6-carboxyfluorescein (FAM), and a 3' quencher, 6-carboxy-*N,N,N',N'*-tetramethylrhodamine (TAMRA; Applied Biosystems). The tick salivary-gland cDNA levels were normalized to *I. scapularis* β -actin gene, and *salp15*, *salp25D* and *B. burgdorferi* N40 flagellin (*flaB*) were then quantified. In the murine infection studies, the mouse β -actin gene was used to normalize the amount of DNA in these samples and *flaB* was used to quantify the levels of spirochaete in the murine tissue samples.

Western blotting. Western blotting was performed as described previously¹¹. The blots were probed with guinea pig Salp15 or Salp25D antisera.

Solid-phase overlay assay. *B. burgdorferi* lysates were separated by conventional SDS-PAGE and transferred by western blotting. The blots were then overlaid with purified recombinant Salp15 (ref. 3). The membranes were probed with horseradish peroxidase-conjugated monoclonal anti-V5 antibody (Invitrogen). Mouse anti-OspC monoclonal antibody was also used to probe the blots.

Confocal microscopy. Salivary glands from nymphal ticks were prepared for confocal microscopy as described previously²⁷. In brief, acetone-fixed glands were incubated with the guinea pig antisera raised against Salp15 and Salp25D, followed by rhodamine-conjugated anti-guinea-pig IgG (Molecular Probes). The glands were counterstained with fluorescein isothiocyanate (FITC)-conjugated goat anti-*B. burgdorferi* antibody (Kirkegaard and Perry Laboratories), and viewed with a Zeiss LSM 510 scanning laser confocal microscope.

Immunofluorescence and *in vitro* binding assay. Wild-type *B. burgdorferi* 297, OspC-deficient and OspC-complemented isolates (10^7 spirochaetes ml^{-1}) were placed on sialyated slides (PGC Scientific). The spirochaetes were then incubated with Salp15-FITC or BSA-FITC³, counterstained with propidium iodide and examined with a Zeiss AxioScope fluorescence microscope.

***In vitro* protection assay.** *B. burgdorferi* N40, OspC-deficient and OspC-complemented isolates (10^8 spirochaetes ml^{-1}) were used. Salp15 was incubated with the spirochaete for 1 h at 25 °C. The spirochaetes were then incubated with OspA monoclonal antibody or polyclonal mouse or rabbit *B. burgdorferi* antisera for 18, 24 or 48 h at 33 °C. The percentage of viable spirochaetes was quantified with the Live-Dead Bacterial viability kit (Molecular Probes).

***In vivo* infection of non-immune mice.** Pathogen-free C3H mice were infected with *B. burgdorferi* (10^2 spirochaetes per mouse) with and without recombinant Salp15 or BSA ($30 \mu\text{g ml}^{-1}$) intradermally. At 25 days after infection, tissue samples were collected for DNA isolation and quantitative PCR.

***In vivo* infection of immune mice.** Pathogen-free C3H mice (five mice per group) were infected with *B. burgdorferi* (10^2 spirochaetes per mouse). At 14 days after infection, the mice were tested for antibody raised against *B. burgdorferi* by enzyme-linked immunosorbent assay. They were then treated with ceftriaxone (16 mg per kg body weight). Ear punches were taken to amplify *flaB* DNA to confirm the absence of spirochaete. The mice were then inoculated with *B. burgdorferi* (10^4 spirochaetes per mouse) with recombinant Salp15 ($30 \mu\text{g}$ per mouse) intradermally. Control mice received *B. burgdorferi* with or without BSA. Tissue samples were collected, and spirochaete levels were quantified 25 days later.

RNA interference. cDNA from nymphs was prepared as described previously and *salp15* was amplified with gene-specific primers 5'-GAGCTCGCATCAA CCGCTGACAAA-3' and 5'-GGTACCCTAACATCCGGGAATGTG-3' containing *SacI* and *KpnI* restriction sites. The *salp15* fragment was cloned into the L440 double T7 Script vector, and the dsRNA was synthesized and purified with the Megascript RNAi kit in accordance with the manufacturer's protocol (Ambion). *salp15* dsRNA was injected as described²⁸ into nymphal *I. scapularis* infected with *B. burgdorferi*. The ticks were then placed on naive or previously infected C3H mice or *P. leucopus* (as described above) and allowed to feed until 72 h. Ticks were collected, cDNA was made from salivary glands and the expression of *salp15* was assessed. Seven days after tick feeding, skin samples from the site of a tick bite were excised and levels of *flaB* were measured with quantitative PCR.

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