

An *Ixodes scapularis* protein required for survival of *Anaplasma phagocytophilum* in tick salivary glands

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***Anaplasma phagocytophilum* is the agent of human anaplasmosis, the second most common tick-borne illness in the United States. This pathogen, which is closely related to obligate intracellular organisms in the genera *Rickettsia*, *Ehrlichia*, and *Anaplasma*, persists in ticks and mammalian hosts; however, the mechanisms for survival in the arthropod are not known. We now show that *A. phagocytophilum* induces expression of the *Ixodes scapularis* *salp16* gene in the arthropod salivary glands during vector engorgement. RNA interference-mediated silencing of *salp16* gene expression interfered with the survival of *A. phagocytophilum* that entered ticks fed on *A. phagocytophilum*-infected mice. *A. phagocytophilum* migrated normally from *A. phagocytophilum*-infected mice to the gut of engorging *salp16*-deficient ticks, but up to 90% of the bacteria that entered the ticks were not able to successfully infect *I. scapularis* salivary glands. These data demonstrate the specific requirement of a pathogen for a tick salivary protein to persist within the arthropod and provide a paradigm for understanding how *Rickettsia*-like pathogens are maintained within vectors.**

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Abbreviations used: dsRNA, double-stranded RNA; GST, glutathione *S*-transferase.

Arthropod-borne infectious diseases are a major threat to human life. Human anaplasmosis, caused by *Anaplasma phagocytophilum*, is an emerging tick-borne illness that has been reported in the United States, Europe, and Asia (1–4). *Ixodes scapularis* is the most common vector in North America. *A. phagocytophilum* is an obligate intracellular pathogen closely related to organisms in the genera *Rickettsia* and *Ehrlichia* that primarily resides within the neutrophils of its mammalian hosts (5). Infection may be self-limiting, or it may result in severe illness or death, particularly in older or immunocompromised individuals.

A. phagocytophilum persists within the secretory salivary acini of tick salivary glands (6). Tick feeding stimulates the replication and migration of the bacteria from the salivary glands to the mammalian host (7). Studies suggest that transmission of *A. phagocytophilum* occurs between 24 (8) and 48 h (9, 10) after tick attachment. Once in the host, *A. phagocytophilum* invades granulocytes and disseminates

(7). It has been shown that the acquisition of *A. phagocytophilum* by uninfected *I. scapularis* larva begins within 2 d of tick attachment on *A. phagocytophilum*-infected mice (9). Once in the tick, *A. phagocytophilum* moves through the gut and infects the salivary glands. Our studies have detected *A. phagocytophilum* within tick salivary glands as early as 24 h after engorgement (unpublished data).

As vector-borne microbes have evolved with arthropods, they have developed an intimate relationship to facilitate survival and transmission. In one strategy, the pathogen attempts to use arthropod or mammalian factors to help complete the microbial life cycle within the vector or reservoir host. As examples, *Borrelia burgdorferi*, the Lyme disease agent, uses a tick salivary protein to help infect the mammalian host (11), and *Leishmania* species use galectins within the sandfly to adhere to the midgut and possibly bind to galectins within the mammalian host (12). *Plasmodium* species may also require carbohydrate-modified ligands to persist

in the mosquito gut and salivary glands (13, 14). The obligate intracellular nature of *Anaplasma* in both the arthropod and mammalian host provides an excellent opportunity to study the factors that are essential for the survival of *A. phagocytophilum* and possibly other *Rickettsia*-like organisms within vectors. In this study, we show that an *I. scapularis* salivary protein, Salp16, is required for the survival of *A. phagocytophilum* within the arthropod salivary glands.

RESULTS

A. phagocytophilum selectively alters the expression of tick salivary gland genes

To study whether *A. phagocytophilum* selectively modulates tick salivary gland gene transcription, the expression of 14 *salp* (salivary protein) genes that encode antigens recognized by the tick-exposed host sera was analyzed in the salivary glands of *A. phagocytophilum*-infected *I. scapularis* nymphs. Salivary glands were dissected at 24 and 72 h of engorgement. Because *A. phagocytophilum* is transstadially transmitted, *A. phagocytophilum*-infected nymphs were produced by feeding larva on *A. phagocytophilum*-infected C3H mice and subsequently allowing them to molt into nymphs. The nymphal infection rate was $82 \pm 13\%$. To generate uninfected nymphs to be used as controls, larvae were fed to repletion on uninfected mice and were permitted to molt.

For the experiments, 25 infected or 25 uninfected nymphs were fed upon three naive C3H mice. Groups of 10 ticks were collected from each mouse at 24 and 72 h after attachment. Tick salivary glands were isolated, RNA was extracted, and PCR was performed to specifically amplify the 14 *salp* genes. Differential expression of several *salp* genes in *A. phagocytophilum*-infected tick salivary glands compared with uninfected glands was noted at both 24 and 72 h after tick attachment (Fig. 1 a). Notably, the expression of *salp16* was most strongly up-regulated in the salivary glands of *A. phagocytophilum*-infected ticks. In contrast, the expression of *salp10*, *salp13*, and *salp17* were down-regulated. Expression of the other *salp* genes, including *salp8*, *salp14*, *salp15*, *salp20*, *salp25A*, *salp25B*, *salp25C*, *salp25D*, *salp26A*, and *salp26B*, was not markedly influenced by *A. phagocytophilum*. The relative change in selected *salp* gene expression upon *A. phagocytophilum* infection was confirmed by quantitative RT-PCR (Table I). The up-regulation of *salp16* was further verified by examining individual salivary glands from a batch of *A. phagocytophilum*-infected ticks rather than pooled samples (Fig. 1 b), and one of many representative examples is presented.

Because the expression of *salp16* was highly up-regulated in *A. phagocytophilum*-infected tick salivary glands, we characterized the temporal relationship of *salp16* induction with tick engorgement and *A. phagocytophilum* infection. For the experiments, five sample groups, each having three pooled salivary glands, were examined at each time point. The samples were taken at precisely the same time point during the feeding cycle. Tick salivary glands were isolated, RNA was extracted, and PCR was performed. First, in the salivary glands of unfed and uninfected ticks, *salp16* was not expressed

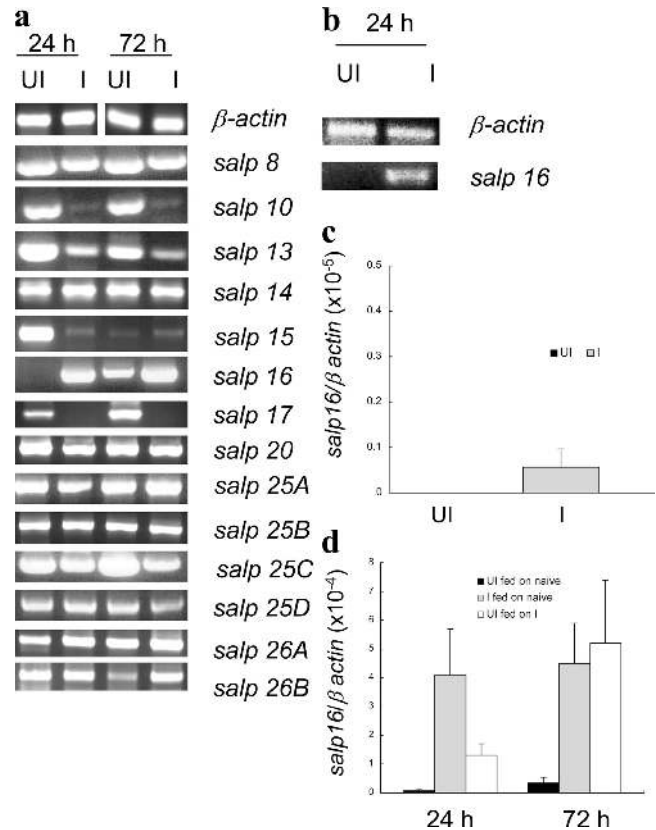


Figure 1. *Anaplasma phagocytophilum* selectively alters *salp* gene expression. (a) The expression levels of 14 *salp* genes in the salivary glands of *A. phagocytophilum*-infected (I) *Ixodes scapularis* nymphs were compared with uninfected (UI) nymphs at 24 and 72 h after feeding using RT-PCR. (b) Verification of the up-regulation of *salp16* expression in *A. phagocytophilum*-infected single tick salivary glands at 24 h after infection by RT-PCR. (c) Expression of *salp16* in the salivary glands of uninfected and infected unfed ticks by quantitative RT-PCR. (d) Expression of *salp16* at 24 and 72 h in the salivary glands of uninfected and infected ticks fed on naive mice or uninfected ticks fed on infected mice by quantitative RT-PCR. Results are expressed as the means \pm SEM (error bars) from three independent experiments. $P < 0.05$ was considered statistically significant (Student's *t* test).

(Fig. 1 c). When uninfected ticks were fed on naive mice, *salp16* expression was barely detectable at 24 h and showed a modest up-regulation at 72 h (fivefold increase from 24 to 72 h; $P < 0.05$), indicating that *salp16* expression is partially induced by feeding, (Fig. 1 d). In the infected unfed ticks, there was a very low level of expression of *salp16* (Fig. 1 c). *salp16* was strongly up-regulated in infected fed ticks (55-fold increase in comparison with uninfected fed ticks; $P < 0.01$) at an early time point (24 h) during engorgement (Fig. 1 d). Moreover, when uninfected ticks were fed on *A. phagocytophilum*-infected mice, *salp16* was up-regulated 18-fold ($P < 0.05$) at 24 h and 15-fold ($P < 0.05$) at 72 h in comparison with that of uninfected ticks that fed on naive mice for identical intervals (Fig. 1 d). It is interesting to note that the *salp16* up-regulation at 72 h, when uninfected ticks engorged on

Table 1. *A. phagocytophilum* selectively alters *salp* gene expression

Gene	Fold change	
	24 h	72 h
<i>salp8</i>	0.9 ± 0.1	0.98 ± 0.03
<i>salp10</i>	0.04 ± 0.01	0.1 ± 0.1
<i>salp13</i>	0.6 ± 0.14	0.3 ± 0.1
<i>salp14</i>	0.98 ± 0.1	1.02 ± 0.01
<i>salp15</i>	0.04 ± 0.001	1.2 ± 0.2
<i>salp16</i>	54 ± 4.5	11.8 ± 1.2
<i>salp17</i>	0.06 ± 0.01	0.03 ± 0.01
<i>salp20</i>	0.9 ± 0.01	0.97 ± 0.1
<i>salp25A</i>	0.99 ± 0.23	1.1 ± 0.3
<i>salp25B</i>	1.04 ± 0.05	1.01 ± 0.1
<i>salp25C</i>	0.9 ± 0.002	0.3 ± 0.04
<i>salp25D</i>	1.1 ± 0.01	1.0 ± 0.02
<i>salp26A</i>	1.2 ± 0.01	1.2 ± 0.1
<i>salp26B</i>	1.01 ± 0.8	3.2 ± 1.2

Fold change of the expression levels of 14 *salp* genes in the salivary glands of *A. phagocytophilum*-infected *I. scapularis* nymphs compared with uninfected nymphs at 24 and 72 h after feeding. Tick β -actin gene expression was used to normalize the amount of cDNA. Results shown are expressed as the ratio of expression levels of each gene (nanograms of cDNA) in infected versus uninfected ticks, which was determined by quantitative RT-PCR analysis.

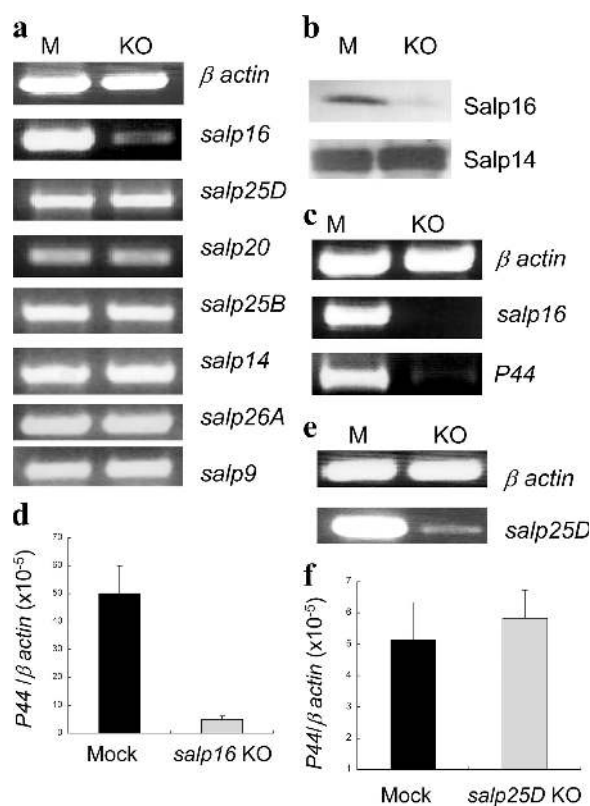
infected mice, was comparable with that observed when infected ticks were fed on naive mice (Fig. 1 d). These data suggest that the presence of *A. phagocytophilum* in the salivary gland during feeding up-regulates *salp16* irrespective of whether *A. phagocytophilum* was already present in the salivary gland or had newly entered the tick during engorgement on an infected mouse (Fig. 1 d). *salp16* mRNA was not detected in the gut of uninfected ticks (15) but was observed upon *A. phagocytophilum* infection (unpublished data).

Silencing of the *salp16* gene using RNAi reduces acquisition of *A. phagocytophilum* by the tick

To examine whether *salp16* plays a role in the survival of *A. phagocytophilum* within ticks, *salp16*-deficient ticks were generated using RNAi. For the construction of *salp16* double-stranded RNA (dsRNA), *salp16* was amplified and cloned from the cDNA of engorged tick salivary glands. Injection of the RNAi construct through the idiosoma of the ticks and the feeding and recovery of *I. scapularis* were performed as described in Materials and methods. There was no visible phenotypic change or impairment in feeding in *salp16*-injected ticks when compared with control buffer-injected ticks.

To assess the efficacy of the RNAi treatment, total RNA isolated from the salivary glands of mock (buffer alone) and *salp16* dsRNA-injected ticks was used as a template to amplify *salp16*. *salp16* mRNA was substantially decreased in the *salp16* dsRNA-injected group (Fig. 2 a), and the percentage of inhibition was $93 \pm 3\%$ as quantified by ImageJ software (National Institutes of Health [NIH]). The expression levels of other *salp* genes, including *salp25D*, *salp14*, *salp20*, *salp25B*,

salp26A, and *salp9*, were not affected by the silencing of *salp16* (Fig. 2 a). The nucleotide sequence of *salp16* is not related to any of the other *salp* genes investigated in this study. The protein extract of mock and *salp16* dsRNA-injected tick salivary glands was analyzed by immunoblotting using recombinant Salp16 antisera to confirm gene silencing at the protein level. Salp16 in salivary glands of ticks injected with *salp16* dsRNA was reduced by $81 \pm 6\%$ (Fig. 2 b). The specificity of gene silencing was determined by probing a duplicate immunoblot with antisera raised against another salivary gland protein, Salp14, and equivalent levels of Salp14 were evident in the control and experimental groups (Fig. 2 b).

**Figure 2.** Silencing of the *salp16* gene using RNAi reduces the acquisition of *Anaplasma phagocytophilum* by the tick.

The efficiency and specificity of RNAi-dependent knockdown of the *salp16* gene was assessed using RT-PCR (a) and immunoblotting (b). (a) The expression of *salp25D*, *salp14*, *salp20*, *salp25B*, *salp26A*, and *salp9* in *salp16*-deficient tick salivary glands. As shown in b, the specificity of knockdown was confirmed using a duplicate immunoblot probed with Salp14 antisera. (c and d) RT-PCR- (c) and quantitative RT-PCR (d)-based demonstrations of the reduced ability of *salp16*-deficient ticks to acquire *A. phagocytophilum* from infected mice. RNAi-injected ticks were fed on infected mice for 72 h, and the level of *A. phagocytophilum* was assessed in the salivary gland using RT-PCR with the *P44* gene as the marker. (e) RT-PCR showing the ablation of *salp25D* after the injection of ticks with a *salp25D* RNAi construct. (f) The acquisition of *A. phagocytophilum* by *salp25D*-depleted ticks. Results are expressed as the mean \pm SEM (error bars) from three independent experiments. $P < 0.05$ was considered statistically significant (Student's *t* test). M, mock; KO, knockout.

We then explored whether the silencing of *salp16* gene expression affects the ability of ticks to acquire *A. phagocytophilum* from the infected mammalian host. Control ticks and ticks injected with *salp16* dsRNA were allowed to feed on each of three *A. phagocytophilum*-infected C3H mice (20 ticks per mice) for 72 h and were then collected. To assess the levels of *salp16* and *A. phagocytophilum* in the salivary glands, RT-PCR was performed using total RNA from mock and *salp16* dsRNA-injected tick salivary glands (from groups of three pairs of glands). The level of the gene encoding a major outer membrane antigen, *P44* of *A. phagocytophilum*, was used to assess the number of viable bacteria. As expected, *salp16* expression was diminished significantly in *salp16* dsRNA-injected ticks (Fig. 2 c). Surprisingly, the level of *A. phagocytophilum* was markedly lower (reduced 10-fold; $P < 0.01$; Fig. 2 d) in *salp16*-deficient ticks. To rule out any bias on *A. phagocytophilum* acquisition by the tick as a result of mouse-to-mouse variations in the bacterial infection, we repeated the acquisition experiments by placing both mock and *salp16*-deficient ticks on the same *A. phagocytophilum*-infected mice. The results were identical to those observed when the acquisition experiment was performed using separate mice for mock and *salp16*-deficient ticks (unpublished data). To further demonstrate that the reduced acquisition of *A. phagocytophilum* by *salp16*-deficient ticks was specific, the acquisition experiment was repeated by silencing another *salp* gene, *salp25D* (Fig. 2 e). *A. phagocytophilum* levels were the same in the mock and *salp25D*-deficient groups (Fig. 2 f).

salp16 silencing does not affect the acquisition of *B. burgdorferi*

We determined whether *salp16* was required for *A. phagocytophilum* acquisition by ticks or were used by another tick-borne pathogen by examining the levels of *B. burgdorferi* in *salp16* dsRNA-injected nymphs after feeding on *B. burgdorferi*-infected C3H mice. *B. burgdorferi* infects the tick gut and moves to the salivary gland immediately before transmission. The guts of engorged ticks were collected at 72 h and examined. As expected, *salp16* expression was markedly reduced in the guts of ticks administered *salp16* dsRNA (Fig. 3 a). Quantitative RT-PCR demonstrated that there was no difference in the level of *B. burgdorferi* in *salp16*-deficient or control ticks (Fig. 3 b). Imaging of the tick gut for *B. burgdorferi* by confocal microscopy yielded similar results (Fig. 3 c).

B. burgdorferi and *A. phagocytophilum* are both transmitted by *I. scapularis* and can sometimes simultaneously infect a host. Therefore, we examined the role of *salp16* in the acquisition of *B. burgdorferi* and *A. phagocytophilum* from coinfecting mice. The acquisition experiment involved feeding 20 *salp16*-deficient ticks on each of the three coinfecting mice. Ticks were collected at 72 h after feeding. Groups of three ticks from each mouse were used to check the levels of *A. phagocytophilum* in the salivary glands and *B. burgdorferi* in the gut. As expected, *salp16* was repressed in the tick salivary glands (Fig. 3 d) and gut (Fig. 3 f). The level of *A. phagocytophilum* was significantly lower in the *salp16*-deficient group in comparison

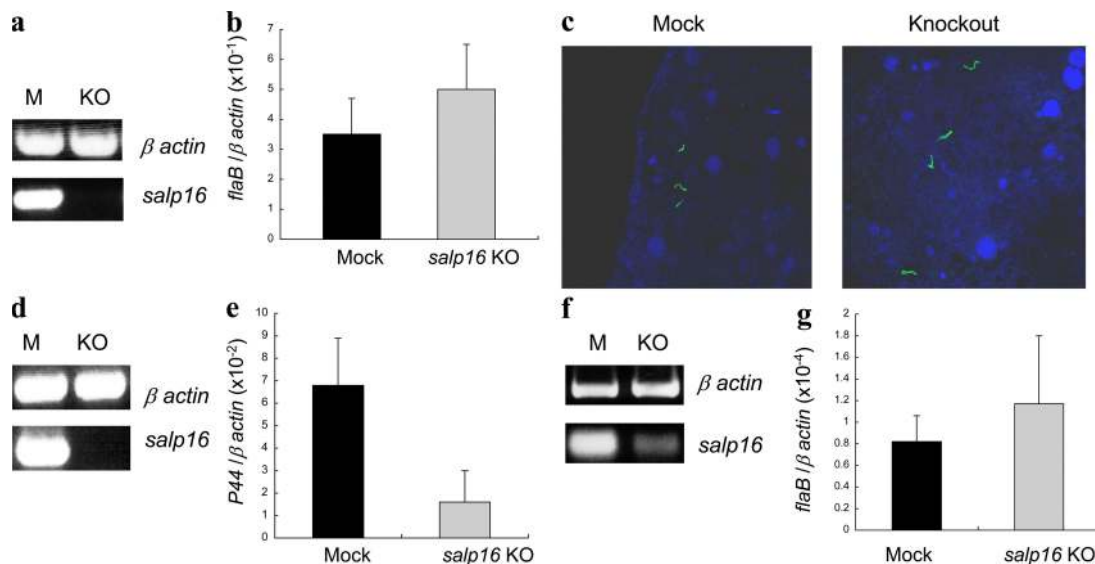


Figure 3. Silencing of *salp16* expression does not affect the acquisition of *B. burgdorferi*. *salp16* RNAi-injected ticks were allowed to feed on *B. burgdorferi*-infected mice, and the guts were analyzed at 72 h after infection. (a) RT-PCR-based confirmation of the silencing of *salp16* in the knockout tick guts. (b) Analysis of the levels of *B. burgdorferi* in the gut of knockout ticks (KO) in comparison with mock-injected ticks (M) by quantitative RT-PCR analysis. *flaB* gene expression was used to measure the levels of *B. burgdorferi*. Results are means \pm SEM (error bars) from one representative experiment. (c) Confirmation of the levels of *B. burgdorferi* in the gut

of mock and knockout ticks by confocal microscopy. Gut samples from mock and knockout ticks were probed with FITC-labeled anti-*B. burgdorferi* antibody (green), and nuclei were stained with TO-PRO. (d) Knockout of *salp16* in the salivary glands and (f) gut of ticks fed on coinfecting mice for 72 h assessed by RT-PCR. The levels of *A. phagocytophilum* (e) and *B. burgdorferi* (g) in ticks fed on coinfecting mice was performed by measuring the levels of *A. phagocytophilum* P44 and *B. burgdorferi* *flaB*. Results are the means \pm SEM from three quantitative PCR experiments. $P < 0.05$ was considered statistically significant (Student's *t* test).

with the mock group ($P < 0.05$; Fig. 3 e), and there was no change in the level of *B. burgdorferi* ($P > 0.05$; Fig. 3 g). Overall, these data show that *salp16* disruption affects the acquisition of *A. phagocytophilum* but not *B. burgdorferi*.

Transmission of *A. phagocytophilum* is not affected in *salp16*-deficient ticks

To examine the role of *salp16* in the transmission of *A. phagocytophilum* from ticks, 10 infected *salp16*-deficient or control nymphs were fed on each of three naive C3H mice. The mice were bled on days 5 and 8 to check the levels of *A. phagocytophilum* in the peripheral blood, time points at which infection is known to be detectable in the bloodstream. Quantitative PCR analysis showed that the bacterial load in the blood of mice fed upon by either control or *salp16*-deficient ticks was similar (copies of *P44* gene per 10^{11} copies of mouse β -actin gene were 2.5 ± 0.8 and 2.3 ± 0.6 on day 5 and 5.9 ± 1.4 and 7.6 ± 1.5 on day 8, respectively), thereby demonstrating that *A. phagocytophilum* does not require *salp16* for infection of the mammalian host.

A. phagocytophilum does not use *salp16* in the mammalian host just before acquisition by the arthropod vector

A. phagocytophilum in mice may be passively recruited to the tick bite site by the chemotaxis of granulocytes (7). It is possible that tick-derived molecules could attract neutrophils and facilitate the entry of *A. phagocytophilum* into the arthropod. Therefore, we examined whether Salp16 interacts with neutrophils, potentially leading to the enhanced entry of *A. phagocytophilum* into ticks. Extracts of human neutrophils did not bind rSalp16 (Fig. 4 a), and rSalp16 did not induce neutrophil chemotaxis (Fig. 4 b). Tick immune sera recognized rSalp16 (Fig. 4 c), and rSalp16 antisera bound native Salp16 (Fig. 2 b), demonstrating that the recombinant and native protein are similar enough to elicit cross-reactive antibodies. The binding assay and the chemotaxis assay were repeated with rSalp16 expressed in *Drosophila* cells, which also showed similar results (unpublished data). rSalp16 expressed in *Drosophila* cells has posttranslational modifications that may not be present in protein expressed in *Escherichia coli*; however, it still may not be identical to native Salp16. We then investigated

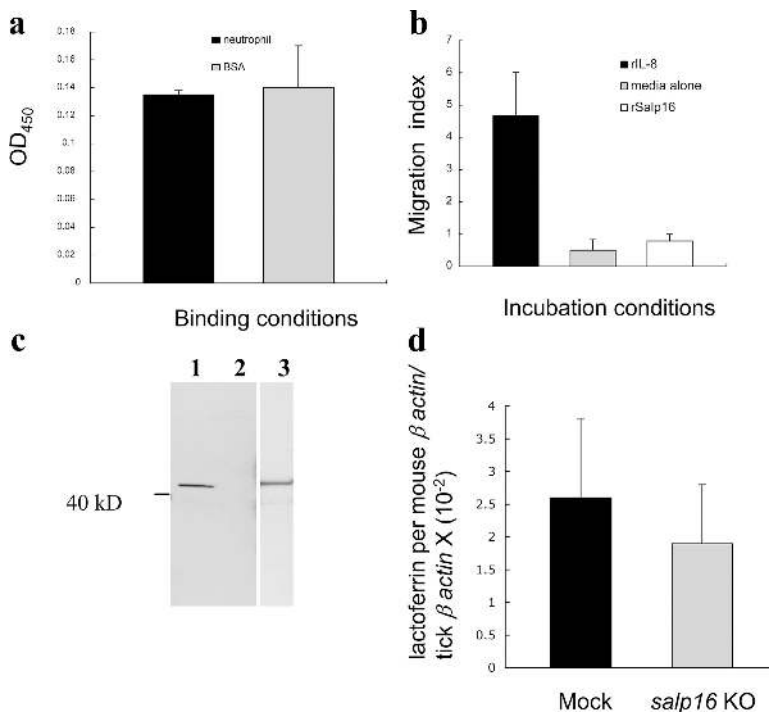
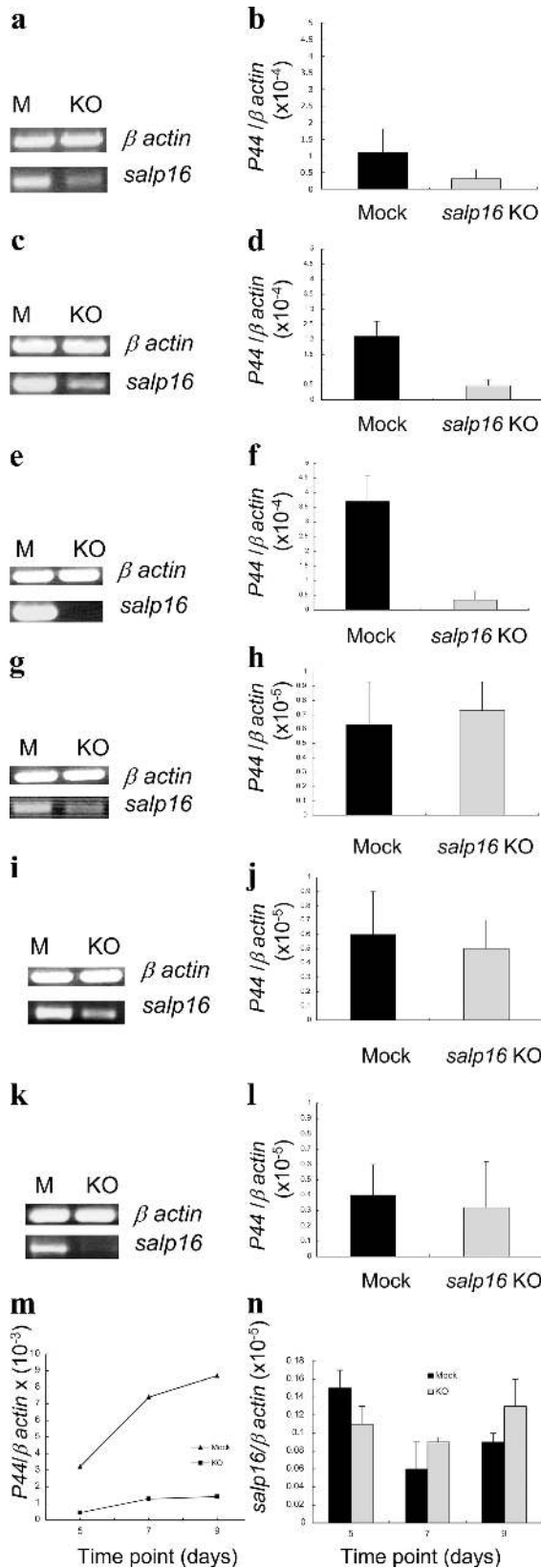


Figure 4. *A. phagocytophilum* does not use Salp16 in the mammalian host before acquisition by the arthropod. (a) Binding of Salp16 with human neutrophils. Extracts of human neutrophils or BSA (control) were incubated with recombinant Salp16, and a binding study was performed as described in Materials and methods. Bars represent the mean \pm SEM (error bars) from three studies. (b) A neutrophil chemotaxis assay using Salp16. Freshly isolated human neutrophils were used as chemoattractants in 96-well Neuro Probe TX chambers together with recombinant IL-8 as a positive control and medium as a negative control as described in Materials and methods. The results are expressed as the migration index after subtraction of background values (medium alone). The means \pm SEM of three independent studies are shown. (c) Immunoblot showing that recombinant Salp16 (expressed in bacteria) is recognized by tick immune sera. Lane 1 shows recombinant Salp16 (GST fusion) probed with rabbit tick immune sera; lane 2 shows GST protein probed with tick immune sera; and lane 3 shows recombinant Salp16 probed with Salp16 antisera. (d) Comparison of the number of neutrophils entering the mock and *salp16* knockout (KO) ticks. After the feeding of mock and *salp16* knockout ticks on infected mice, the number of neutrophils within the whole ticks was assessed by measuring the *lactoferrin* (neutrophil-specific gene) levels (normalized to mouse β -actin gene and tick β -actin gene) by quantitative RT-PCR analysis. The results are expressed as means \pm SEM from three independent experiments.

(c) Immunoblot showing that recombinant Salp16 (expressed in bacteria) is recognized by tick immune sera. Lane 1 shows recombinant Salp16 (GST fusion) probed with rabbit tick immune sera; lane 2 shows GST protein probed with tick immune sera; and lane 3 shows recombinant Salp16 probed with Salp16 antisera. (d) Comparison of the number of neutrophils entering the mock and *salp16* knockout (KO) ticks. After the feeding of mock and *salp16* knockout ticks on infected mice, the number of neutrophils within the whole ticks was assessed by measuring the *lactoferrin* (neutrophil-specific gene) levels (normalized to mouse β -actin gene and tick β -actin gene) by quantitative RT-PCR analysis. The results are expressed as means \pm SEM from three independent experiments.



whether the presence of Salp16 enhances the acquisition levels of neutrophils by ticks. Neutrophil-specific marker-based PCR analysis of *salp16*-deficient and wild-type ticks after feeding showed equal numbers of neutrophils, demonstrating that neutrophil entry into ticks is independent of Salp16 (Fig. 4 d). These experiments support the hypothesis that the functional requirement of *A. phagocytophilum* for Salp16 occurs within the vector alone.

A. phagocytophilum requires Salp16 for initial infection of the tick salivary gland

We then determined whether Salp16 is involved in the entry of *A. phagocytophilum* to the tick gut or salivary gland. A time course assessment of the level of *A. phagocytophilum* in the gut and salivary glands of feeding ticks assessed whether Salp16 is involved in the ingress of the bacteria into these tissues. Mock or *salp16*-deficient ticks were placed on each of three *A. phagocytophilum*-infected C3H mice (20 ticks on each mice), and, at 24, 48, and 72 h after feeding, three ticks from each group were collected. Salivary gland and gut samples were analyzed for the level of *salp16* and *A. phagocytophilum*. Fig. 5 (a, c, and e) shows the repression of *salp16* in the salivary glands at 24, 48, and 72 h. Similarly, Fig. 5 (g, i, and k) represents the repression of *salp16* in the gut at 24, 48, and 72 h. There was a significant reduction in the levels of *A. phagocytophilum* in the salivary glands of *salp16*-deficient ticks at 24 ($P < 0.05$; Fig. 5 b), 48 ($P < 0.05$; Fig. 5 d), and 72 h ($P < 0.01$; Fig. 5 f). The relative reduction of *A. phagocytophilum* in *salp16*-deficient glands compared with bacteria in mock glands gradually increased from 73 to 90% as the feeding time increased from 24 to 72 h. In contrast, the levels of *A. phagocytophilum* in the gut did not show any change at any time points when mock ticks were compared with *salp16* dsRNA-injected ticks ($P > 0.05$; Fig. 5, h, j, and l). The presence of similar numbers of *A. phagocytophilum* in the gut at all intervals indicates that *salp16* is not required by the bacteria in the gut. Fig. 5 m shows that at later postfeeding time points (days 5, 7, and 9), the relative reduction of *A. phagocytophilum* in *salp16*-deficient salivary glands compared with mock glands were 86.3, 82.8, and 83.9% ($P < 0.05$), respectively. However, as

Figure 5. *A. phagocytophilum* requires Salp16 for the initial infection of the tick salivary gland. A time course assessment of the levels of *A. phagocytophilum* within the salivary glands and guts of *salp16*-deficient (KO) and mock (M) ticks fed on infected mice for 24, 48, and 72 h is shown. Repression of *salp16* in the salivary glands of ticks fed for 24 (a), 48 (c), and 72 h (e) by RT-PCR. The levels of *A. phagocytophilum* P44 within the salivary glands of ticks fed for 24 (b), 48 (d), and 72 h (f) by quantitative RT-PCR. Repression of *salp16* within the corresponding gut samples of ticks fed for 24 (g), 48 (i), and 72 h (k) by RT-PCR. The levels of *A. phagocytophilum* P44 in the gut samples of ticks fed for 24 (h), 48 (j), and 72 h (l). (m) Levels of *A. phagocytophilum* P44 in the salivary glands of ticks on days 5, 7, and 9 after feeding, and (n) levels of *salp16* in the salivary glands at the respective time points. The results are expressed as means \pm SEM (error bars) from a representative experiment. $P < 0.05$ was considered statistically significant (Student's *t* test).

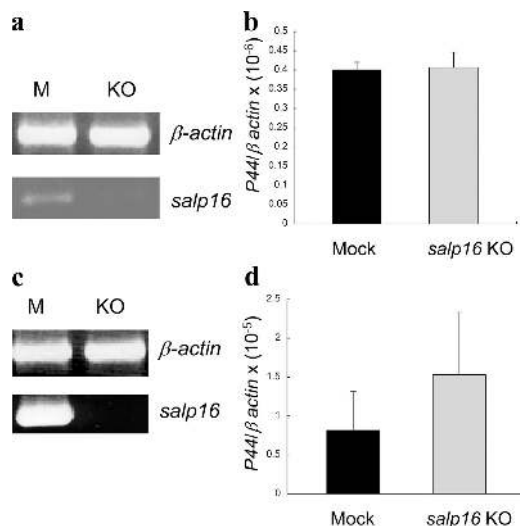


Figure 6. *salp16* is not required for the maintenance of *A. phagocytophilum* within infected tick salivary glands. *salp16* RNAi was injected into *A. phagocytophilum*-infected ticks and were analyzed either in an unfed state or in a 72-h fed state. (a) Knockdown of *salp16* expression in *A. phagocytophilum*-infected unfed ticks by RT-PCR, and (b) quantification of *A. phagocytophilum* P44 levels by quantitative RT-PCR analysis. (c) Knockdown of *salp16* expression in 72-h fed ticks by RT-PCR, and (d) quantitative RT-PCR data showing *A. phagocytophilum* P44 levels. Results are means \pm SEM (error bars) from three independent experiments. $P < 0.05$ was considered statistically significant (Student's *t* test). M, mock; KO, knockout.

indicated in Fig. 5 n, the expression level of *salp16* in both mock and *salp16*-deficient ticks were barely detectable at these time points and were comparable with its levels in the salivary glands of unfed infected ticks. The level of *A. phagocytophilum* was substantially lower in the salivary glands of *salp16*-silenced ticks in comparison with mock salivary glands at all time points examined, suggesting that *A. phagocytophilum* specifically requires *salp16* to infect salivary glands.

We then studied the role of *salp16* in the survival of *A. phagocytophilum* within a previously infected salivary gland. We monitored the levels of *A. phagocytophilum* in the salivary glands of infected nymphal ticks (infected as larvae and molted to nymph) in which *salp16* was subsequently repressed. The *A. phagocytophilum*-infected *salp16*-deficient ticks were examined either in an unfed state (Fig. 6 a) or after 72 h (Fig. 6 c) of feeding. There was no significant difference in the levels of *A. phagocytophilum* when we compared mock with *salp16* dsRNA-treated groups ($P > 0.05$; Fig. 6, b and d). These results show that *salp16* is not required for the maintenance of *A. phagocytophilum* in the salivary glands of an infected tick.

DISCUSSION

Ixodes ticks transmit many important human and veterinary pathogens, including *B. burgdorferi* (16), *A. phagocytophilum* (6), *Babesia microti* (17), *Bartonella* species (18), and tick-borne encephalitis virus (19). A tick receptor that enables *B. burgdorferi* to attach to the tick gut and, thereby, infect *I. scapularis*

has been recently identified (20). However, the arthropod ligands participating in pathogen survival in ticks and tick salivary glands, particularly of the related group of obligate intracellular microbes that include *Anaplasma*, *Rickettsia*, *Ehrlichia*, and *Neorickettsia*, are unknown (21). In this study, we demonstrate that *A. phagocytophilum* requires an *I. scapularis* protein, Salp16, to infect tick salivary glands.

The striking temporal relationship between *A. phagocytophilum* infection, tick feeding, and the strong up-regulation of *salp16* first raised the possibility that *A. phagocytophilum* may need this protein within the vector. The lack of a notable induction of *salp16* in an unfed *A. phagocytophilum*-infected tick compared with an unfed uninfected tick (in contrast with the robust stimulation of *salp16* during *I. scapularis* engorgement) suggested that *A. phagocytophilum* does not require *salp16* to persist once infection of the tick has been established. Indeed, when *salp16* expression was knocked down in a tick previously infected with *A. phagocytophilum* (infected as a larvae and molted to nymph), there was no difference in the level of *A. phagocytophilum* within salivary glands compared with that of control ticks. In contrast, the acquisition of *A. phagocytophilum* from the infected mammalian host was severely inhibited (up to a 10-fold reduction from controls) in *salp16*-deficient ticks. Collectively, these data show a specific dependence of *A. phagocytophilum* on Salp16 for the microbe's successful acquisition by *I. scapularis*.

We then determined when in the *A. phagocytophilum* life cycle Salp16 was necessary. Ticks may secrete Salp16 into mice during the process of engorgement because Salp16 protein has been found in tick saliva by antigen-capture ELISA (15, 22). Moreover, Salp16 antibodies have been identified in animals exposed to repeated tick bite (15, 22), and Salp16 has a putative secretory signal sequence, as assessed by the SignalP 3.0 program (www.cbs.dtu.dk/services/SignalP/), with the cleavage of the signal peptide predicted to occur between amino acid residues 18 and 19. We also showed that tick immune sera, which is raised against secreted tick proteins, recognizes rSalp16 (Fig. 4 c). The immunostaining of Salp16 in the engorged tick salivary glands using anti-rSalp16 antisera shows that Salp16 is distributed throughout the salivary gland (Fig. 7), similar to the pattern of two other secreted salivary gland proteins, Salp14 and Salp25D (23). We performed studies to examine whether *A. phagocytophilum*-Salp16 interactions could be important in the host environment. These data (Fig. 4) suggested that *A. phagocytophilum* does not use Salp16 in the host just before acquisition by the vector. Therefore, we focused on delineating the stage of *A. phagocytophilum* life cycle within *I. scapularis* that is influenced by Salp16. Temporal acquisition studies (Fig. 5, c, e, i, and k) in *salp16*-depleted ticks showed a marked reduction (up to 90%) in the levels of *A. phagocytophilum* within *salp16*-deficient *I. scapularis* salivary glands compared with controls. In contrast, the levels of *A. phagocytophilum* within the gut of *salp16*-deficient and control ticks remained the same at all time periods, demonstrating that Salp16 is not required by *A. phagocytophilum* in the gut. A postfeeding time course analysis

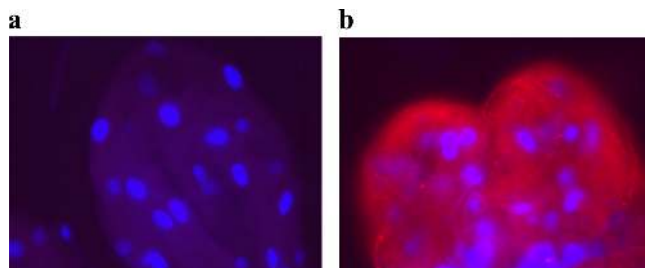


Figure 7. Confocal microscopy showing the distribution of Salp16 within engorged tick salivary glands. Ticks were fed for 72 h, and salivary glands were isolated and probed with anti-GST antisera (negative control; a) and anti-Salp16 polyclonal antisera (b). Binding was visualized using tetramethylrhodamine isothiocyanate-conjugated secondary antibody, and the samples were counterstained with nuclear stain DAPI.

showed that the reduction in the level of *A. phagocytophilum* within the salivary glands of *salp16*-deficient ticks was consistently maintained throughout the 9-d examination period (Fig. 5 m). Collectively, these results show that Salp16 is required by *A. phagocytophilum* for the infection of *I. scapularis* salivary glands.

I. scapularis ticks also vector *B. burgdorferi*, the causative agent of Lyme disease and the most common tick-transmitted bacterium in North America (16). Coinfections and co-transmissions of *B. burgdorferi* and *A. phagocytophilum* are known to occur (24). Thus, there was an interest to examine whether Salp16 was also critical for the acquisition of *B. burgdorferi*. *B. burgdorferi* infection did not alter the expression level of *salp16* in ticks (unpublished data). Furthermore, RNAi knockdown experiments (Fig. 3 b) demonstrated that Salp16 was not essential for *B. burgdorferi* acquisition. The observation that the abrogation of *salp16* impairs the acquisition of *A. phagocytophilum* and not *B. burgdorferi* from a coinfecting mouse host (Fig. 3 g) underscores the functional importance of Salp16 in *A. phagocytophilum* infection. *I. scapularis* ticks can also harbor and transmit *B. microti* (17). Like *A. phagocytophilum*, *B. microti*, a protozoan pathogen, infects the salivary gland of ticks. However, unlike *A. phagocytophilum*, *B. microti* infection did not alter the expression of *salp16* (unpublished data). Further testing with various tick-borne pathogens will determine whether *salp16* is used by any other pathogens.

The mechanism by which Salp16 enables *A. phagocytophilum* to infect *I. scapularis* salivary glands remains to be elucidated. The reduction in the level of *A. phagocytophilum* in the salivary glands of *salp16*-deficient ticks compared with control ticks as feeding progresses suggests that Salp16 may be required by the bacterium during an early stage of infection of the salivary glands, perhaps at entry or immediately after entry. In addition, the postfeeding (>72 h) multiplication rates of *A. phagocytophilum* within the salivary glands of mock and *salp16*-deficient ticks were similar (Fig. 5 m), further suggesting that the pathogen requires Salp16 early during infection. Finally, the integrity of the vacuolar membrane

surrounding the *A. phagocytophilum* morulae within neutrophils is critical for its survival (25), and it is logical to expect that the *Anaplasma*-containing vacuole within the arthropod is also vital for bacterial growth. This vacuole could certainly contain both tick and bacterial components. Salp16 could be directly or indirectly involved in this process in the salivary glands; however, *Anaplasma*-containing vacuoles are likely to also be important in the gut, where Salp16 does not appear to be essential. It is conceivable that during tick feeding and pathogen acquisition, the bacterium may be predominantly contained in morulae within the host neutrophils entering the gut along with the blood meal. As *A. phagocytophilum* leaves these morulae, poised to infect tick salivary glands, the role of Salp16 may be vital. To date, little is known of how *A. phagocytophilum* transitions from the host neutrophil to the tick salivary glands.

Obligate intracellular pathogens in the genera closely related to *Anaplasma* are among the most poorly understood pathogens despite their historical and current importance. As examples, *Rickettsia prowazekii*, the agent of epidemic typhus, has been a leading cause of death during war and famine throughout history (26). Rocky mountain spotted fever, caused by *Rickettsia rickettsia*, has recently been described in new locations in the United States (27), and *A. phagocytophilum* is now the second most common tick-borne disease in North America. These data now identify a vector molecule that is essential for the survival of *A. phagocytophilum* within *I. scapularis* and serves as a paradigm for understanding how obligate intracellular pathogens may survive within arthropods. It is likely that related agents may also require specific vector antigens to successfully complete their life cycle. The molecular aspects of arthropod-pathogen interactions are only beginning to be explored (11, 12, 28). The identification of these fundamental interactions will enable us to develop new methods to interrupt the pathogen-vector-host cycle and may lead to novel methods to prevent or contain disease.

MATERIALS AND METHODS

Mice. 4–6-wk-old female C3H/HeN and C3H/SCID mice were purchased from the NIH and The Jackson Laboratory, respectively. The maintenance of mice and the animal experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee at the Yale University School of Medicine.

Infection of C3H/HeN mice with *A. phagocytophilum* and/or *B. burgdorferi*. The *A. phagocytophilum* isolate NCH-1 (6) used in these studies was maintained through serial passage of infected blood in C3H/SCID mice as described previously (29). Quantitative PCR was performed on an aliquot of the pooled blood collected from the SCID mice to quantitate and standardize the infectious dose of *A. phagocytophilum*.

For the acquisition experiments, C3H/HeN mice were injected intraperitoneally with 100 μ l of pooled *A. phagocytophilum*-infected or uninfected (controls) anticoagulated blood. The bacterial burden in the peripheral mouse blood was assessed on day 6. Total genomic DNA was isolated from the peripheral blood using the DNeasy tissue kit (QIAGEN) according to the manufacturer's protocol, and the levels of *P44* were measured using quantitative PCR. Ticks were then allowed to engorge upon the infected

mice on day 7, the interval of maximal infection of *A. phagocytophilum* in the peripheral blood.

B. burgdorferi isolate N40 was used to infect C3H/HeN mice (10⁴ bacteria per mouse). 2 wk after infection, the mice were tested by taking ear punches to amplify *flaB* DNA in order to confirm the presence of spirochetes. DNA extraction and PCR analysis were performed as described previously (20).

To generate coinfecting mice, the C3H/HeN mice were first challenged with 10⁴ *B. burgdorferi* followed by challenge with *A. phagocytophilum* on day 7. On day 14, after the infection with *B. burgdorferi* and 7 d after infection with *A. phagocytophilum*, the mice were tested for the presence of both *B. burgdorferi* and *A. phagocytophilum* as described above.

Ticks. I. scapularis nymphs and larvae and *B. burgdorferi*-infected nymphs (70% infected) were obtained from continuously maintained tick colonies. To generate *A. phagocytophilum*-infected nymphs, the larvae were fed to repletion on *A. phagocytophilum*-infected C3H/HeN mice and molted into nymphs. To produce uninfected nymphs (controls), larvae engorged on uninfected mice and were then allowed to molt. Tick rearing was conducted in an incubator at 23°C with 85% relative humidity and a 14/10 h light/dark photo period regimen. 10% of the molted nymphs from each infection group were individually tested by PCR to confirm infection and to determine prevalence. The infection rate was 82 ± 13% for *A. phagocytophilum*-infected nymphs.

RNAi. Salivary glands isolated from engorged nymphs were used to isolate cDNA. *salp16* was amplified using the primers S16RNAiF (5'-GAAGATCTGACTCGCTGGTTTATGTTTC-3') and S16RNAiR (5'-GGGGTACCATTGTCTTTGTTACTGTTGC-3') containing BglII and KpnI restriction enzyme sites. The amplified *salp16* fragment was cloned into the L4440 double T7 Script II vector, and dsRNA was synthesized using the Megascript RNAi kit (Ambion). 5 nl (5 × 10¹² molecules/μl) *salp16* dsRNA was injected into uninfected *A. phagocytophilum*- or *B. burgdorferi*-infected nymphal ticks. The dsRNA was inoculated through the idiosoma of the ticks into the hemocoel. The feeding and recovery of ticks were performed as previously described (23). The control ticks (mock) received 0.5 nl of the injection buffer (10 mM Tris-HCl, pH 7.5, and 1 mM EDTA). Ticks fed for the respective time points were collected, cDNA was made from the salivary glands, and the expression of *salp16* was assessed using primers 16F-RNIC (5'-CTGAAGTTCCTTTATCTCTTC-3') and 16R-RNIC (5'-GCA-GGGTCCTTCTCGGG-3'). The construction of *salp25D* RNAi was performed in a similar fashion using primers *salp25DRNAiF* (5'-AGATCTCCACGAATGGCTCGGC-3') and *salp25DRNAiR* (5'-GGTACCGGAACAGCTTGAGAATC-3'). The *salp25D* RNAi injection, feeding of ticks, and cDNA synthesis from the salivary glands were performed as described for *salp16*. The expression of *salp25D* after the RNAi experiment was assessed using primers *salp25DRT-F* (5'-CCTTCCCCAACTTACC-3') and *salp25RT-R* (5'-GTCCATGGTTGTTCCGAG-3').

Immunoblots. Salivary glands (10 pooled samples) isolated from the mock and *salp16*-deficient ticks were resuspended in sterile PBS with protease inhibitors (protease inhibitor cocktail; Sigma-Aldrich) and homogenized. Equal amounts of salivary gland protein (10 μg) from mock or *salp16* dsRNA-injected ticks were electrophoresed on an SDS/12% polyacrylamide gel and processed for immunoblotting. The immunoblots were probed with recombinant Salp16 antisera. A duplicate immunoblot was probed with recombinant Salp14 antisera. For the immunoblot showing tick immune sera recognizing recombinant Salp16 (glutathione S-transferase [GST] fusion), 1 μg of bacterial recombinant Salp16 and 1 μg GST protein were electrophoresed on an SDS/12% polyacrylamide gel and probed with rabbit tick immune sera. As a positive control, rSalp16 was probed with anti-Salp16 antisera. The bound antibodies were detected by using horseradish peroxidase-conjugated rabbit anti-mouse and goat anti-rabbit secondary antibodies (Sigma-Aldrich), and the blots were developed using the enhanced chemiluminescence Western Blotting Detection System (GE Healthcare).

Acquisition experiment. For the acquisition experiments, control ticks and ticks injected with *salp16* dsRNA were allowed to feed on *A. phagocytophilum*-infected, *B. burgdorferi*-infected, or coinfecting C3H/HeN mice for 72 h and were collected. We used three mice in each group (experimental and control/mock), and 20 ticks were placed on each mouse. cDNA was made from pooled salivary glands or guts of three fed ticks. After the confirmation of the knockdown of *salp16*, the levels of the *P44* gene of *A. phagocytophilum* and the *flaB* gene of *B. burgdorferi* were measured by quantitative RT-PCR to assess the levels of viable bacteria. For the time point acquisition experiments, mock and *salp16* dsRNA-injected ticks were fed for 24, 48, and 72 h on *A. phagocytophilum*-infected C3H/HeN mice, and salivary glands (pooled in groups of three pairs) from each group were collected at each time point and analyzed as described for the 72-h acquisition experiments. To determine the levels of *A. phagocytophilum* in the salivary glands of mock and *salp16* dsRNA-injected ticks at later time points (days 5, 7, and 9), the engorged ticks were maintained in an incubator at 23°C with 85% relative humidity, and the salivary glands (three pairs pooled) were dissected on the indicated days.

To rule out the possibility of any bias on *A. phagocytophilum* acquisition by the tick as a result of variation in the infection levels in various mice, we also performed an acquisition experiment with single mice. In these experiments, the mock group of nymphs was injected with buffer containing 1 nM Ponceau S (Sigma-Aldrich). 20 ticks (*salp16* dsRNA injected or mock injected) were placed on a single mouse, allowed to feed for 72 h, and collected. The Ponceau S dye stained the injection site pink and enabled us to distinguish mock-injected and dsRNA-injected ticks.

Transmission experiment. *A. phagocytophilum*-infected and *salp16*-deficient or control nymphs (10 ticks each) were allowed to feed on each of three naive C3H/HeN mice. Once the ticks fed to repletion, the mice were bled on days 5 and 8. Total genomic DNA was isolated from the peripheral blood using the DNeasy tissue kit (QIAGEN), and the level of *P44* was measured using quantitative PCR.

RNA extraction, cDNA synthesis, RT-PCR, quantitative RT-PCR, and genomic DNA isolation. Total RNA was extracted from the salivary glands and gut samples using TRIzol reagent (Invitrogen). The RNA was treated with TURBO DNase (Ambion) to remove the contaminating DNA. The purified RNA was used for RT-PCR and quantitative PCR analysis.

cDNA was made from the tick salivary glands and gut samples using the iScript cDNA synthesis kit (Bio-Rad Laboratories). Quantitative PCR was performed using an iCycler Real-Time Detection System (Bio-Rad Laboratories). Real-time PCR was performed according to the iQ SYBR Green Supermix (Bio-Rad Laboratories) protocol. The relative gene expression levels of each transcript were determined by comparison with a standard curve. The salivary gland or gut cDNA levels were normalized to the *I. scapularis* β -actin gene, and the levels of *P44* and *flaB* genes were then quantified (iCycler IQ software version 3.1; Bio-Rad Laboratories). In the mouse infection studies, the mouse β -actin gene was used to normalize the levels of DNA in the samples, and *P44* was used to assess the levels of *A. phagocytophilum* in the mouse blood samples. The sequence-specific primers for the *P44* gene were HF (5'-CTACTAGCTAAGGAGTTAGC-3') and HB (5'-CAGAGAAGTAGAAGAAACCG-3'; reference 30) and for the *flaB* gene were FlabF (5'-TTCAATCAGGTAACGGCACA-3') and FlabR (5'-GACGCTTGAGACCCTGAAAG-3'). The primers used for RT-PCR, quantitative RT-PCR to analyze the expression of 14 *salp* genes, and their GenBank accession numbers are as described previously (15, 22).

To quantify neutrophil entry into mock and *salp16*-deficient ticks during acquisition feeding, total genomic DNA was isolated from whole ticks that had fed for 72 h using the DNeasy tissue kit (QIAGEN). The expression level of a neutrophil-specific gene, *lactoferrin*, was used as a direct readout of the number of neutrophils entering the ticks during feeding. Equal amounts of total tick genomic DNA (0.5 μg) from either mock or *salp16*-deficient ticks were used as a template to amplify the mouse neutrophil *lactoferrin* gene using the primers LACTMF (5'-CGGACTCACTACTATGC-3') and

LACTMR (5'-TCCTCAAGGGATGCAGGT-3'), and the levels of DNA were normalized to the mouse β -actin gene and to the tick β -actin gene.

Protein expression and preparation of polyclonal antibody. *salp16* was amplified using primers 5'-CCGCTCGAGGATACAAGTCCCAGTGAG-ACAGGA-3' and 5'-ATAAGAATGCGGCCGCATGCAGCTTGGCAA-GGG-3' and was cloned into pGEX6P-1 vector (GE Healthcare) for bacterial expression or into pIZ/V5/His vector (Invitrogen) for *Drosophila* expression. In BL21 bacterial cells, the *salp16* gene was induced with 1 mM isopropyl- β -D-thiogalactoside for 3 h at 37°C, and the culture pellet was lysed using a French press. After centrifugation at 7,500 g for 30 min, the supernatant was allowed to pass through a glutathione-Sepharose 4B column (GE Healthcare), washed extensively, and eluted. The glutathione transferase fusion protein tag was cleaved using PreScission Protease (GE Healthcare). *Salp16* was expressed in a *Drosophila* cell-based system and purified using Talon affinity chromatography (CLONTECH Laboratories, Inc.). The antibody against bacterially expressed *Salp16* was raised in both mice and rabbit using standard protocols.

In vitro binding assay. Human neutrophils were isolated as described previously (31, 32). The binding assays of r*Salp16* to neutrophil extracts were performed as previously described (33). In brief, 0.5 μ g of neutrophil extract was coated on ELISA plates overnight at 4°C. After blocking the nonspecific sites, the wells were incubated with r*Salp16* (1 μ g/well) for 1 h followed by incubation with r*Salp16* antisera. Binding was detected using anti-mouse IgG-horseradish peroxidase conjugate as secondary reagent, and TMB Microwell peroxidase substrate was used for color development. The OD was measured at 450 nm.

Neutrophil chemotaxis assay. 2×10^6 freshly isolated human neutrophils suspended in 50 μ l of Hank's balanced salt solution (Invitrogen) were added to the upper compartment of a 96-well ChemoTx System Chemotaxis Chamber (Neuro Probe, Inc.). 1 μ g/well r*Salp16* was added to the lower compartment. 0.1 μ g/well of human recombinant IL-8 was used as the positive control and the medium alone as the negative control. After washing off the cells attached under the filter after 30 min of incubation at 37°C with 5% CO₂, the migrated cells were determined by reading the absorbency at 490 nm after the addition of CellTiter 96 Aqueous assay reagent (Promega). The OD values of the samples were expressed relative to the standard curve values, yielding an index of cells that migrated across the membrane.

Confocal microscopy. The levels of *B. burgdorferi* within the mock and *salp16*-deficient tick gut samples were analyzed by confocal microscopy as described previously (34). In brief, the acetone-fixed gut samples were incubated with FITC-conjugated anti-*B. burgdorferi* antisera (Kirkegaard and Perry Laboratories, Inc.) followed by the nuclear stain TO-PRO (Invitrogen). Staining of *Salp16* within tick salivary glands was performed as previously described (23). Acetone-fixed salivary gland samples were incubated with control GST antisera and anti-*Salp16* antisera, respectively. The binding was visualized using tetramethylrhodamine isothiocyanate-conjugated secondary antibody. The samples were counterstained with the nuclear stain DAPI and viewed with a scanning laser confocal microscope (LSM 510; Carl Zeiss MicroImaging, Inc.).

Statistical analysis. Results are expressed as the means \pm SE. The significance of the differences between the mean values of two groups of mice or time periods was evaluated by Student's *t* test. Calculated *P* values of <0.05 were considered significant.

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