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Adela S. Oliva Chávez
Xiaowei Wang
Liron Marnin
Nathan K. Archer
Holly L. Hammond

See next page for additional authors

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Tick extracellular vesicles enable arthropod feeding and promote distinct outcomes of bacterial infection

Adela S. Oliva Chávez 1,18, Xiaowei Wang 1, Liron Marnin 1, Nathan K. Archer 2, Holly L. Hammond 1, Erin E. McClure Carroll 1,19, Dana K. Shaw 1,20, Brenden G. Tully 3, Amanda D. Buskirk 4,21, Shelby L. Ford 5, L. Rainer Butler 1, Preeti Shahi 1, Kateryna Morozova 6, Cristina C. Clement 6,22, Lauren Lawres 7, Anya J. O’Neal 1, Choukri Ben Mamoun 7, Kathleen L. Mason 8, Brandi E. Hobbs 1, Glen A. Scoles 8,23, Eileen M. Barry 4, Daniel E. Sonenshine 9,10, Utpal Pal 11, Jesus G. Valenzuela 9, Marcelo B. Sztein 4,12,13, Marcela F. Pasetti 4,12, Michael L. Levin 5, Michail Kotsyfakis 8, Steven M. Jay 15, Jason F. Huntley 3, Lloyd S. Miller 2,16, Laura Santambrogio 17,22 & Joao H. F. Pedra 1

Extracellular vesicles are thought to facilitate pathogen transmission from arthropods to humans and other animals. Here, we reveal that pathogen spreading from arthropods to the mammalian host is multifaceted. Extracellular vesicles from *Ixodes scapularis* enable tick feeding and promote infection of the mildly virulent rickettsial agent *Anaplasma phagocytophilum* through the SNARE proteins Vamp33 and Synaptobrevin 2 and dendritic epidermal T cells. However, extracellular vesicles from the tick *Dermacentor andersoni* mitigate microbial spreading caused by the lethal pathogen *Francisella tularensis*. Collectively, we establish that tick extracellular vesicles foster distinct outcomes of bacterial infection and assist in vector feeding by acting on skin immunity. Thus, the biology of arthropods should be taken into consideration when developing strategies to control vector-borne diseases.
**Results**

**Ticks secrete salivary effectors within diverse EVs.** We developed a methodology for recovering tick EVs from cells and organs in an EV-depleted medium (Supplementary Fig. 1a, b). EVs from the blacklegged tick *I. scapularis* were heterogenous, as shown by transmission electron microscopy (Fig. 1a). In silico reconstruction of the EV pathway based on the *I. scapularis* genome identified genes associated with exosome biogenesis and secretion (Supplementary Data 1). These molecules included the tetraspanin CD63 and two proteins associated with the endosomal sorting complex required for transport (ESCRT) machinery: (i) α-1,3/1,6-mannosyltransferase interacting protein X (ALIX) and the (ii) tumor susceptibility gene 101 protein (TSG101)15–17. Mammalian polyclonal antibodies cross-reacted with markers present in EVs originating from three tick cell lines: (i) *Amblyomma americanum* (i.e., vector of ehrlichiosis37–39 and AAE2 cells); (ii) *I. scapularis* (i.e., vector of Lyme disease and Anaplasmosis44–45–ISE6 cells); and (iii) *D. andersoni* (i.e., vector of tularemia48 – DAE100 cells) (Fig. 1b and Supplementary Fig. 1c–e). These EVs presented an average mean size of 173 ± 7, 137 ± 6, and 183 ± 3 nm, respectively (Supplementary Fig. 1f–h and Supplementary Movies 1–3).

To study the effect of *I. scapularis* EVs, we silenced the expression of two tick soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) genes (e.g., synaptobrevin 2 and vam33) through RNA interference (RNAi). Tick studies rely on delivering small interfering RNA (siRNA) to modulate gene expression because complete genetic ablation within ticks is not currently feasible39. No differences in cell viability were observed between the scrambled (sc) control and silenced (si) treatments (Supplementary Fig. 2a). Nanoparticle tracking analysis (NTA) revealed decreased secretion of tick vesicles in the classical exosomal range (i.e., 50–150 nm) for both synaptobrevin 2 and vam33 siRNA-treated cells (Fig. 1c, Supplementary Fig. 2b–d, and Supplementary Movies 4–7).

EVs derived from adult *I. scapularis* salivary glands had an average mean size of 198 ± 4 nm (Fig. 1d and Supplementary Movie 8). We then obtained EVs from dissected *I. scapularis* salivary glands cultured ex vivo in a vesicle-free medium to analyze their protein content. High-throughput proteomics analysis of their cargo demonstrated a heterogeneous population with proteins typically featured for microvesicles, exosomes, and exomeres40 (Supplementary Fig. 2e, f, Supplementary Data 2–5, and data available via ProteomeXchange, identifier PXD018779). Tick proteins that have an impact on the immune response (e.g., sialostatins9 and molecules associated with oxidative stress, metabolism, and cell biology processes were overrepresented (Supplementary Figs. 3 and 4 and Supplementary Data 2). The presence of the exosomal markers CD63, ALIX, and TSG101 was detected in tick salivary EVs by immunoblots (Fig. 1e–g), as shown previously41. Moreover, a subset of proteins detected in our proteomics analysis was validated through western blots (Fig. 1h).

Some proteins were present in all EVs surveyed (e.g., DHX16 and PL3), while others were only detected in EVs originated from tick cells (e.g., CTNNB1) when compared to salivary glands (e.g., CCT7 and GFPPT1). A similar pattern emerged for glycosylation in tick EVs (Fig. 1i), but this observation was less pronounced for phosphorylation (Fig. 1j) and carbonylation (Fig. 1k), a common product of protein oxidation42.

It was not possible to corroborate all our findings with EVs from tick saliva due to the limited material. Due to this technical limitation, we adopted a hybrid isolation mode where we substantiated our results with EVs from tick saliva in *I. ricinus*, the main vector of Lyme disease in Europe43. This methodology was also recently used in EVs extracted from *I. scapularis* and *Amblyomma maculatum* to determine their effect on a human keratinocyte cell line44. Using this approach, we identified the tick protein Sialostatin L2 (SL2)9 and the tetraspanin CD63 in EVs derived from adult saliva (Fig. 1l). Altogether, our data suggested that ticks secrete a diverse group of EVs containing molecules capable of modulating mammalian host physiology.
Tick EVs interact with mammalian immune cells. We then postulated that molecules present in EVs bind to immune cells. Proteomics of *I. scapularis* EVs derived from salivary glands revealed an overrepresentation of proteins connected to cell biology, oxidative stress, and metabolism (Fig. 2a, b, Supplementary Figs. 3 and 4, and Supplementary Data 2 and 5). Integrins direct EV tropism to specific organs through molecular interactions in the extracellular matrix. To test this concept, we incorporated a fluorescent dye with long aliphatic tails (PKH26) into lipid regions of membranes from tick EVs. We also labeled the plasma membrane of murine bone marrow-derived macrophages (BMDMs) pre-treated with cytochalasin D, an inhibitor of actin polymerization, to block phagocytosis. Laser scanning confocal microscopy coupled to live-cell imaging showed PKH26-labeled EVs bound to murine BMDMs (Fig. 2c and Supplementary Movie 9) and time-lapse microscopy displayed saturation at ~160 min (Fig. 2d). Next, we labeled *I. scapularis* EVs with the lipophilic 3,3′-dioctadecyloxacarbocyanine (DiO) dye and murine
BMDMs with the marker F4/80 conjugated to allophycocyanin (APC). F4/80+ murine macrophages bound to tick EVs in a dose-dependent manner (Fig. 2e, f). Similarly, tick EVs bound to CD11b+ human macrophages originated from peripheral blood mononuclear cells (PBMCs) (Fig. 2g, h). Collectively, our results indicated that arthropod EVs interact with immune cells.

Tick EVs redirect skin immunity. To assess whether tick EVs regulate an immune environment in vivo, we evaluated skin cell populations during *I. scapularis* hematology. Under normal tick-feeding, we observed increased inflammation in the murine skin, as inferred by an elevated presence of granulocytic and phagocytic cells and greater epidermal thickness (Supplementary Fig. 5). These findings were consistent with tick bites eliciting a visible innate immune response in the skin of humans. Next, we performed RNAi silencing of vamp33 and synaptobrevin 2 in *I. scapularis* in vivo. For the sake of simplicity, we denominated arthropods that had vamp33 or synaptobrevin 2 gene expression reduced (vamp33 or synap2 si) as EV-deficient ticks. No difference in tick attachment was observed in the silenced versus the control treatment (Fig. 3a and Supplementary Fig. 6a, b). However, diminished feeding was measured for EV-deficient ticks (Fig. 3b and Supplementary Fig. 6c). Impaired tick feeding was linked to a change in the cytokine and chemokine milieu in the mouse skin. Notably, the skin immune environment was skewed towards an adaptive T helper 2 (Th2) wound healing response in mice fed on by EV-deficient ticks (Fig. 3c–e and Supplementary Fig. 7). Surprisingly, there was a 54% increase in the number of gamma delta (γδ) T cells when EV-deficient ticks were placed on mice and compared to the control treatment (Fig. 3f and Supplementary Fig. 8). Increased numbers of γδ T cells at the skin site correlated to favored chemotactic conditions for immune cell recruitment during a tick bite (Fig. 3g–j and Supplementary Fig. 7).

Depletion of DETCs rescues EV-dependent tick feeding at the skin site. γδ T cells were the only immune cells that showed an increased at the skin site after impairment of tick EVs (Fig. 3f and Supplementary Fig. 8). Thus, we determined the effect of skin γδ T cells on tick feeding. We first reduced the number of γδ T cells in the murine skin through antibody depletion (Fig. 4a). We placed ticks injected with vamp33 siRNA on mice and measured their weight at day 3 post-attachment. We detected a 24% reduction in the weight of EV-deficient ticks for the isotopic treatment (Fig. 4b, weight, two left-most rectangular bars [Antι-γδ Ab]). This decrease in weight was not observed when EV-deficient ticks (vamp33 si treatment) were placed on antibody-depleted γδ T cell mice (Fig. 4b, weight, two right-most rectangular bars [Anti-γδ Ab]). Similar results were obtained in animals genetically ablated for γδ T cells (Fig. 4c, weight, comparison in the two left-wildtype [WT] and right-most rectangular bars [tcrδ−/−] mice). Importantly, αβ T cells did not play any role in arthropod feeding. The weight of EV-deficient ticks (vamp33 si treatment) placed on animals genetically ablated for αβ T cells was reduced when compared to the control treatment (Fig. 4c, weight, comparison in the middle [tcrδ−/−] mice). Taken together, our findings suggested that EVs regulate skin immunity through γδ T cells, providing an advantageous environment for tick feeding.

We then determined whether the effect of tick EVs was on resident or infiltrating γδ T cells. We placed ticks injected with vamp33 siRNA on mice with or without administering the molecule FTY720. FTY720 inhibits lymphocyte egress from lymph nodes. We did not observe any differences in the number of γδ T cells or the weight of EV-deficient ticks placed on FTY720-treated mice when compared to the control treatment (Fig. 4e). These findings indicated that tick EVs affect the resident, but not the infiltrating γδ T cells during feeding.

Tick saliva may be injected in the epidermis, dermis, or the epidermis/dermis border depending on the tick mouthpart apparatus. The γδ T cell population in the mouse skin is constituted of two distinct subpopulations, including dermal γδ T cells (also known as γδ T17 cells) that express the T cell receptor Vγ4 or Vγ6,28,30, and DETCs that express the T cell receptor Vγ5Vδ1 in the epidermis, according to the Heilig and Tonegawa nomenclature. Thus far the effect of the tick bite has been predominantly reported to the dermis. Therefore, we first...
investigated dermal γδ T cells in the context of tick EV secretion. Dermal γδ T cells rely on the chemokine receptors CCR2 and CCR6 for cell recruitment to inflammatory sites and CCR2 for homeostatic trafficking\(^\text{28,29}\). We placed ticks injected with vamp33 siRNA on ccr2\(^{-/-}\) and ccr6\(^{-/-}\) mice. Surprisingly, genetic ablation of CCR2 or CCR6 did not affect EV-dependent tick feeding (Supplementary Fig. 9), suggesting that the dermal γδ T cells were not involved in tick feeding mediated by EVs.

Subsequently, we examined the role of (Vγ5+Vδ1+) DETCs during tick feeding\(^\text{28,29}\). We observed a greater thickening of the mouse epidermis during a tick bite (Supplementary Fig. 5b), which correlated with the epidermal function of DETCs\(^\text{28,29}\). We placed ticks injected with vamp33 siRNA on wild-type mice and measured tick weight at day 3 post-attachment. We detected a significant increase in frequency of DETCs during a tick bite in the vamp33 siRNA treatment when compared to vamp33 scRNA control ticks (Fig. 4f). These results also agreed with our flow cytometry data showing the majority of γδ T cells in the skin bitten by the tick were TCRγδhi (Fig. 4g). Conversely, the predominant population of γδ T cells in the draining lymph nodes were TCRγδlo, which are non-Vγ5+Vδ1+ T cells. Altogether, these findings strengthened our argument that DETCs are the important γδ T cell subset in the skin during a tick bite.

Then, we investigated the effect of DETCs on tick feeding by placing ticks injected with vamp33 siRNA on FVB-Tac mice. FVB-Tac mice are naturally depleted of DETCs due to a failure of thymic selection because of a natural mutation of the skint1 gene\(^\text{52}\) (Fig. 4h, left-most rectangular bars). A decrease in weight was not observed when ticks deficient in EVs were placed on FVB-Tac (DETC depleted) mice (Fig. 4h, right-most rectangular bars). Conversely, we detected the opposite findings in FVB-JAX (DETC-sufficient) mice, which do not bear a mutation in the skint1 gene and harbored normal levels of DETCs. Collectively, we discovered that tick EVs regulate DETCs in the epidermis for an optimal feeding environment.

### Intracellular bacteria manipulate the release of tick EVs during infection

As I. scapularis regulated host immunity, we examined whether intracellular bacteria altered the release of tick EVs upon infection. Accordingly, we detected a modified morphology and heterogeneity of EVs originating from tick cells previously infected with the intracellular rickettsial bacterium A. phagocytophilum, but not the extracellular spirochete B. burgdorferi (Fig. 5a and Supplementary Movies 10–12). Consistently, neither infection of tick cells with B. burgdorferi nor the human parasite Babesia microti substantially increased the secretion of EVs (Fig. 5b, Supplementary Fig. 10a, and Supplementary Movies 13 and 14). However, tick cells released more EVs in the classical exosomal range upon infection with the intracellular bacteria Ehrlichia chaffeensis and F. tularensis (Supplementary Fig. 10b, c and Supplementary Movies 15–18). Overall, we discovered that tick cells modify the production of EVs according to the intracellular bacterial lifestyle.

Oxidative stress has been recently linked to the pentose phosphate pathway (PPP) via the protein nuclear related factor 2 (NRF2)\(^\text{53}\). Previously, we indicated that cells change their oxidation state and produce reactive oxygen species during A. phagocytophilum infection\(^\text{54}\). We observed an enrichment of proteins from the PPP and the NRF2 signaling network in EVs derived from I. scapularis salivary glands (Fig. 5c, Supplementary Figs. 3 and 4, and Supplementary Data 2 and 4). Therefore, we asked whether the altered cargo content of tick EVs during bacterial infection could be related to the oxidation of protein side chains (e.g., carbonylation)\(^\text{42}\). We incubated EVs from I. scapularis cell lines 1SE6 and IDEl2 with 2,4-dinitrophenylhydrazine (DNPH), which selectively binds to carbonyl groups\(^\text{55}\). Carbonylation present in tick EVs were then probed with an anti-
DNPH antibody after derivatization. Interestingly, carboxylated proteins were detected in tick EVs at much higher levels in the A. phagocytophilum-infected treatment compared to the control group (Fig. 5d, e). A similar effect was not observed for glycosylation (Fig. 5f) or phosphorylation (Fig. 5g). Collectively, we found out that the intracellular bacterial lifestyle modifies the cargo content and carboxylation within tick EVs.

**Tick EVs enable distinct outcomes of bacterial infection in the mammalian host.** We then asked whether tick EVs could affect microbial infection to the mammalian host. We tested this hypothesis by using two distinct models: (i) *F. tularensis*, which causes sepsis followed by death in mice56, and (ii) the mildly virulent rickettsial bacterium *A. phagocytophilum*, which does not cause lethality in mice54. We observed that *F. tularensis* infection via the intradermal route, which mimics tick infection, was fully virulent (Fig. 6a, b)56. Isolation of EVs from partially fed *D. andersoni* ticks, the vector of *F. tularensis* (agent of tularemia) in the western United States38, showed a heterogeneous EV population with an average mean size of 204 ± 6 nm (Fig. 6c and Supplementary Movie 19). We then investigated whether EVs from *D. andersoni* ticks were protective against *F. tularensis*-induced sepsis. Mice injected with *D. andersoni* EVs and *F. tularensis* had also lessened splenomegaly (Fig. 6f) and decreased levels of interferon (IFN)-γ and tumor necrosis factor (TNF)-α in the blood (Fig. 6g and Supplementary Fig. 11a). Surprisingly, bacterial numbers were reduced in the liver and the spleen, but not the lungs of mice injected with *F. tularensis* and *D. andersoni* EVs (Fig. 6h, i and Supplementary Fig. 11b).

Finally, we developed an animal model of *F. tularensis* infection in mice using the tick *D. variabilis*. The intent was to determine whether injecting 10⁷ colony-forming units (CFU) of *F. tularensis* into mice was an experimentally judicious approach. This animal model of tick infection considered the 5-day blood meal of *D. variabilis* and the rapid lethality of *F. tularensis*. Surprisingly, *D. variabilis* harbored a very large amount of *F. tularensis* with an average of 9.5 × 10⁵ CFU/tick after repletion, to a high of 6.5 × 10⁶ CFU/tick of *F. tularensis* on week 4 post-feeding prior to molting (Fig. 6i). Following these results, we then assessed *D. variabilis* infection of *F. tularensis* in the laboratory onto naïve mice. Examination of mouse blood revealed that 100% of naïve mice were infected with *F. tularensis* on day 14 after tick placement (Fig. 6k). We observed high bacterial burdens in the blood (6 × 10⁵ CFU/mL), livers, lungs, and spleens (3 × 10⁵ – 1.9 × 10⁶ CFU/mg tissue). Importantly, our results corroborated with a reproducible and quantitative model of *F. tularensis* in *D. variabilis*57,58 and revealed that the presence of *F. tularensis* inside...
ticks was comparable to what has been described on Martha’s Vineyard\textsuperscript{59}. Martha’s Vineyard is an island off the coast of Massachusetts where epizootic transmission of tularemia by ticks has occurred since 2001\textsuperscript{60}.

Opposite findings were observed when the mildly virulent bacterium \textit{A. phagocytophilum} and \textit{I. scapularis} EVs were injected intradermally into animals. \textit{I. scapularis} EVs favored establishment of \textit{A. phagocytophilum} to mice (Fig. 7a, b). No differences in cytokine release were observed between treatments during \textit{A. phagocytophilum} infection (Fig. 7c, d). Collectively, our findings show that tick EVs distinctly regulate the infection of \textit{A. phagocytophilum} and \textit{F. tularensis} in the mammalian host during feeding.

**Discussion**

In most infectious diseases, the clinical outcome of an illness is determined primarily by interactions between the pathogen and the mammalian host. Nevertheless, for vector-borne diseases, ailment is not only driven by the arms race between the pathogen and the host, but also the arthropod. Currently, arthropod EVs are thought to enable pathogen transmission to mammals\textsuperscript{18,19}. Here, we indicate that microbial infection mediated by arthropod EVs is more intricate than previously observed. Tick EVs promoted infection of the mild rickettsial agent \textit{A. phagocytophilum} to the mammalian host. Conversely, tick EVs decreased \textit{F. tularensis} morbidity and mortality in mice. Importantly, tick EVs...
Danae variabilis nymphs were placed onto naïve female C3H/HeN mice on day -5 and allowed to feed for three days. Mice were intravenously infected on day 0 with 1 × 10^7 CFU of Francisella tularensis. EVs were harvested from D. variabilis saliva glands and from the extracellular milieu made by a multiplex cytokine ELISA and plaque assays at day 5 post-infection. Data is presented as a mean and standard error of the mean (±SEM). Statistical significance was determined using a two-tailed t test of Francisella and Francisella + EVs. f p = 0.03; g p = 0.0004; h p = 0.05; i p = 0.0017. j D. variabilis nymphs were placed onto naïve female C3H/HeN mice on day -5 and allowed to feed for three days. Mice were intravenously infected on day −2 with 1 × 10^7 CFU of F. tularensis. Engorged (repleted) ticks and the mouse blood were collected 2 days later (day 0) and the CFU were obtained at indicated time points. The graph is representative of two independent experiments. k Infected adult D. variabilis ticks (week 14) were individually placed onto naïve mice to examine F. tularensis infection. Ticks completed their blood meal by day 8 and the mouse blood was harvested to quantitate bacterial numbers. Data is presented as mean ± SEM. Five mice were monitored through day 18 with animals being euthanized when moribund. Skull and bones denote a single mouse death, whereas skull and bones with the pound sign indicates death of two mice. Survival was analyzed with the Kaplan–Meier curve. Statistical analysis was performed with the a, d Log-rank (Mantel-Cox) or f-i the two-tailed t test. In b, e statistical analysis of weight data was not done due to the differential animal mortality during experiment. EVs extracellular vesicles, CFU colony-forming units. *p ≤ 0.05. NS not significant. Source data are provided as a Source Data file.

acted locally on skin immunity. The observation that tick EVs inhibited IFN-γ and TNF-α in the mouse blood during F. tularensis infection was likely a consequence of molecular cues initiated at the skin site and distally propagated to other organs.63, Furthermore, the effect of D. andersoni EVs in the spleen and the liver of mice injected with F. tularensis was an outcome of disease in our animal model.

Over the past century, researchers have debated the temporal kinetics of tick transmission of F. tularensis and the onset of tularemia in animal models.62–64. Unfortunately, there is no consensus or universal approach that broadly describes the F. tularensis concentration inside the tick and in the saliva during transmission. Different tick infection techniques, environmental versus laboratory experiments, animal models, tick species, developmental stages, and Francisella genotype/strains lead to distinct experimental outcomes and disagreements among investigators. Thus, additional analysis will be required to determine the relevance of our findings in the natural habitat of ticks.

Correlative evidence suggested the role of SNARE proteins in arthropod feeding.65–68 SNAREs are involved in the fusion of multivesicular bodies to the plasma membrane, releasing EVs into the extracellular milieu.65–67. The scientific community has not yet firmly developed technical approaches to visualize and quantify EVs in vivo.65–71. To date, only a few laboratories around the world have developed fluorescent proteins that are tagged to the plasma membrane to observe EVs in vivo by intravital microscopy or other sophisticated imaging techniques.69,70. Additionally, these technologies are not yet viable in ticks because there are not any genome editing tools for I. scapularis.39 Finally, tick feeding and salivation occur as an intermittent process for several days on a mammalian host,71 and this uneven feeding activity changes the composition of molecules within the saliva72,73. Thus, the quantification of EVs or microbial numbers secreted into the bite site during feeding is not feasible at this time. Given these impediments, we determined a reasonable number of EVs to use in our assays. Experimental approaches varied widely in the literature with EVs ranging from 10^6 to 10^8 molecules.19–47. Hence, we concluded that the administration of 1 × 10^8 EVs was a reasonable approach in our animal model.

The skin contains many immune cells that transport salivary antigens to the draining lymph nodes.49 Most studies have portrayed the effects of the tick bite as taking place within the local feeding cavity and in the dermis.4,49. However, an earlier study revealed antigens that may be trapped in the skin and affect an area beyond the dermal–epidermal location.72 For I. scapularis, the effect of EVs was dependent on γδ T cells. γδ T cells act as a bridge between innate and adaptive immunity28–30, and are important against infection by tick-borne pathogens.78,79. γδ T cells also detect tissue damage and are involved in wound repair.28–30. We observed that inhibition of EV biogenesis in I.
scapularis} ticks resulted in a significant increase of γδ T cells at the bite site. Remarkably, feeding has also been shown to reduce the number of circulating γδ T cells in animals infested with the ticks *Haemaphysalis bispinosa* and *Hyalomma anatolicum*.

*I. scapularis* EVs affected DETCs, which are located in the mouse epidermis and important for wound healing.

**Methods**

**Reagents and resources.** All primers, reagents, resources, manufacturers, and catalog numbers are listed in Supplementary Data 6 and 7.

**EV-depleted medium.** L15C500 medium was supplemented with 5% fetal bovine serum (FBS; MilliporeSigma), 5% tryptose phosphate broth (TPB; BD), 0.1% lipoprotein concentrate (LPC; MP Biomedicals), 0.25% sodium bicarbonate (NaHCO₃; MilliporeSigma), and 25 mM HEPES (MilliporeSigma).

**Ticks.** *I. scapularis* ticks were obtained from three independent sources: (1) the Centers for Disease Control and Prevention through the Biodefense and Emerging Infectious Diseases (BEI) Resources Repository; (2) Dr. Utpal Pal at the University of Maryland, College Park; and (3) Dr. Ulrike Munderloh and Dr. Jonathan Oliver at the University of Minnesota. Unfed nymphs and partially engorged adult female ticks were maintained in an I-30BL incubator (Perical Scientific) at 25 °C under saturated humidity (>85%) in 16:8 h light/dark cycle. *D. variabilis* ticks were housed in 3-dram plastic vials in glass desiccators, with 12-h light and 100% saturated humidity (>85%) in 16:8 h light/dark cycle.

**Blood.** Skin and blood samples were collected from BALB/c, C57BL/6J, and FVB/Jax mice. Skin samples were collected with a scalpel at 3 days post-infection. Blood samples were collected by cardiac puncture at 3 days post-infection. Skin samples and blood were collected from mice infected with *A. phagocytophilum*. The presence of *A. phagocytophilum* in skin and blood was confirmed by real-time PCR and multiplex ELISA, respectively.
Cell lines. *L. scapularis* (ISE6), D. andersoni (DAE100), and *A. americanum* (AAE2) embryonic derived cell lines were obtained from Dr. Ulrike Munderloh at the University of Minnesota through a material transfer agreement. Tick cell lines were maintained in Cellstar™ 25 cm² flasks (Greiner bio-one), containing L15C300 medium supplemented with 5% FBS, 5% TPB, and 0.1% LPC kept at 34 °C.

Microbial infection. *A. phagocytophilum* strain HZ was cultured in 75 cm² flasks (CytoOne) containing HL-60 cells. Briefly, HL-60 cells were cultured in 20 ml of RPMI medium, supplemented with 10% Fetal Bovine Serum and 1x Glutamax. A. phagocytophilum infected cells (500 µl) were added to 5 ml of uninfected cells at 1 to 5 x 10⁶ cells/ml diluted in 24.5 ml of media. The percentage of infection was monitored by the Richard-Allan Scientific™ three-step staining (Thermo Fisher Scientific). Infected cells were spun onto microscope slides with a Cytospin 2 (Shandon). Cells were visualized by light microscopy with an Axioskop microscope (Zeiss). Bacteria were purified once cultures had reached >70% infection. Bacterial numbers were estimated using the number of infected HL-60 cells × 5 morulae/cell × 19 bacteria/cell × 0.5 (representing 50% recovery rate). Infected cells were concentrated by centrifugation at 10,000×g for 5 min. Cells and bacteria were resuspended in 5 ml of L15C300 EV-free medium. Bacteria were isolated from cells through a 27-G bent needle. Cell debris was separated from bacteria by centrifugation at 600×g for 10 mins. The supernatant was collected, and bacteria were then centrifuged at 10,000×g for 5 mins. Pelleted bacteria were resuspended in L15C300 EV-free media and inoculated into confluent ISE6 25 cm² flasks (Greiner bio-one) at a 50:1 MOI.

Tick saliva. Tick saliva was collected as described by Hackenberg et al. Briefly, fully fed *Ixodes ricinus* females (6–7 days) were fixed onto glass slides. Salivation was induced by applying 2 µl of pilocarpine solution (33 g/l in 96% ethanol; Sigma-Aldrich) to the tick dorsal scutum. Saliva was collected into capillary tubes placed around the hypostome of each tick. Ticks were maintained within a humidified chamber in sterile conditions at 37 °C for 2–3 h during salivation. The saliva was pooled, filtered through a 0.22-µm filter, and stored in an 80 °C before being shipped to the University of Maryland School of Medicine.

**Mice.** Experiments were performed on C57BL/6 (WT) and C57BL/6J (fl/fl) mice at 6 weeks of age. All animal procedures were approved by the Animal Care and Use Committee of the University of Maryland School of Medicine and were performed in accordance with the guidelines of the United States Institutional Animal Care and Use Committee.

Tick salivary gland culture. Salivary gland EVs were purified from ex vivo culture originated from partially engorged *I. scapularis* adult females and nymphs. Adult *I. scapularis* females were fed on 6–10-month-old female New Zealand white rabbits for 5–6 days at the CDC facilities and then shipped to the University of Maryland School of Medicine. For the nymph salivary gland culture, *I. scapularis* nymphs were fed for 3 days on C57BL/6 WT mice. *D. andersoni* females were fed on Holstein cows for 5–6 days inside of stockyards. The tick glands were still in moated blocks. After 24 h, ticks were placed on the skin and allowed to feed for 5 days. Partially-fed adult female ticks (25–69) and nymphs (15–30) were dissected 1–2 days post-removal. Ticks were dissected with 4 mm vannas scissors (Fine Scientific Tools) under a Stereo Zoom stereoscope (Bausch and Combs). In all, 10 µl of PBS were added to samples to avoid desiccation. Midguts, Malpighian tubes, and other organs were removed. Salivary glands were dissected and cultured in 24-well cell culture plates (Corning). 10 salivary glands from adult ticks or 20 salivary glands from nymphs were placed in each well, containing 500 µl of L15C300 EV-free medium supplemented with 1x penicillin/streptomycin and 20% FBS. Salivary glands were incubated for 24 h at 34 °C to allow EV secretion. To study the effect of intracellular bacteria on the content of salivary EVs, salivary glands (70–80) from adult female ticks (35–40) were dissected, as described above. Ten salivary glands were placed per well in vesicle-free medium supplemented with Amphotericin B (Gibco). Cell-free *A. phagocytophilum* was isolated and inoculated to the salivary cultures (1.4 x 10⁶ bacteria/well). Salivary glands were incubated for 24 h at 34 °C in the presence or absence of bacteria.

**EV purification.** Medium collected from cell or salivary gland cultures were cleared of any live cells by centrifugation at 300 × g for 10 min at 4 °C. Dead cells were removed by a second centrifugation at 2000 × g for 10 min at 4 °C. The supernatant was collected and apoptotic bodies were removed by a third centrifugation at 10,000 × g for 30 min at 10 °C. To reduce the number of EVs >200 nm in size, the supernatant was filtered through a 0.22-µm Millipore syringe filter (Millipore Sigma). EVs were pelleted by ultracentrifugation (100,000 × g) for 18 h at 4 °C. Supernatant was discarded and EVs were resuspended in PBS. Salivary EVs were purified using a protocol modified from Michael et al. and Zlotogorski-Hurvitz et al. In brief, tick saliva was centrifuged at 1300 × g for 10 min at 4 °C to discard any cells present in the sample. The supernatant was collected and centrifuged for 20 min at 12,000 × g at 4 °C. Following this procedure, the recovered material was diluted 1:1 in 1x PBS to reduce viscosity and centrifuged at 17,000 × g for 15 min at 4 °C. The supernatant was collected and the EVs were purified by ultracentrifugation at 100,000 × g for 18 h at 4 °C. EVs were resuspended in RIPA buffer for western blotting.

Transmission electron microscopy. ISE6 cells were infected with either *A. phagocytophilum* or *B. burgdorferi*. EVs from equal number of infected or uninfected cells (4.4 x 10⁶) were purified as described above and resuspended in PBS. EVs were further concentrated using Amicon Ultra 0.5 ml centrifugal filters 30 K (Millipore-Sigma). Samples were glow-discharged with 400 mesh formova-coated copper grids (Electron Microscopy Sciences) and negatively stained with 1% uranyl acetate in 1% sodium pyrophosphate (UA). These grids were examined in a Tecnai T12 transmission electron microscope (Thermo Scientific) at an operating voltage of 80 kv. Images were acquired using a bottom mount CCD camera (Advanced Microscopy Techniques) and AMT600 software (Advanced Microscopy Techniques). Transmission electron microscopy images were analyzed using ImageJ.

**EV quantification.** EV concentration and sizes were determined using the NanoSight LM10 or NS300 (Malvern Panalytical) machines with NTA software versions 2.0 or 3.0, respectively. The mean of the size generated in the NTA reports was used to calculate the average size of the EVs in each sample. Data was analyzed using GraphPad Version 9.1.0 from Prism.
in 25 mM ammonium bicarbonate buffer. Proteins were digested with trypsin and Lys-C mix (Promega) at 37 °C overnight. Following digestion, samples were evaporated to 100 µl and desalted and concentrated with a C18 ZipTip (Millipore).

**NanoLC–ESI–MS/MS analysis of tryptic/Lys-C peptides.** Peptides were sequenced and analyzed by nanoLC–ESI–MS/MS using QExactive HD quadrupole orbitrap mass spectrometers (Thermo Fisher Scientific), which was coupled to an Easy nLC 1000 UPLC (Thermo Fisher Scientific) through a nanoelectrospray ion source. Peptides were separated on a C18 analytical column (100 µm internal diameter, × 20 cm length) packed with 2.7 µm Phenomenex Cortecs particles. The solution of peptides was equilibrated with 3 µl 5% acetonitrile 0.1% formic acid, before being separated using optimized 180 min linear gradients employing 2–32% acetonitrile in a buffer composed of 0.1% formic acid in water (Buffer A) and 0.1% formic acid in 100% acetonitrile (Buffer B) (Optima LC/MS; Fisher Scientific). Data acquisition was performed using the instrument provided Xcalibur® (version 3.0) software. The mass spectrometer was operated in the positive ionization and data-dependent acquisition (DDA) mode. The full MS scans were obtained with a range of m/z 300 to 2000, at a mass resolution of 120,000 at m/z 200, and a target value of 1.00E+06 with the maximum injection time of 50 ms. HCD collision was performed on the 15 most significant peaks, and tandem mass spectra were acquired at a mass resolution of 15,000 at m/z 200 and a target value of 1.00E+05 with the maximum injection time of 110 ms. The dynamic exclusion time was 20 s. The normalized collision energy was optimized for 32–34%.

**Protein identification.** Raw files were “de novo” sequenced and assigned with a protein ID using the Peaks 8.5/X-software (Bioinformatics Solutions, Waterloo, Canada) by searching against the “I. scapularis” genome in SwissProt database (Jan 2020) and the above mentioned analysis was validated manually using the following criteria: (1) series of at least at least 3 or 4 significant peaks (which uses the decoy fusion method). Protein identifications were accepted if they could be assigned a confident score (q-value) >0 and −10lgP)>20 for peptides (corresponding to p-value < 1.0E−5). The ionization and data-dependent acquisition of the MS+1 was performed in the positive ion mode using an ESI source. The MS/MS fragment ions were analyzed using the optimized X!Tandem algorithm, and then searched against SwissProt and uniprot databases (version 3.0) software. The mass spectrometer was operated in the positive ionization and data-dependent acquisition (DDA) mode. The full MS scans were obtained with a range of m/z 300 to 2000, at a mass resolution of 120,000 at m/z 200, and a target value of 1.00E+06 with the maximum injection time of 50 ms.

**Functional analysis.** Proteins were annotated with GO terms from NCBI (downloaded on March 2016). Networks, functional analyses, and biochemical and cellular pathways were generated by employing the ingenuity pathway analysis (IPA; Ingenuity Systems) (Supplementary Data 4). For all IPA analysis, we used the identified proteins with their corresponding IDs presented in Supplementary Data 2. These molecules were overlaid on a global molecular network provided by the Ingenuity Knowledge Base. The gene-ontology (GO) enrichment, networks and canonical pathways were then algorithmically generated based on their connectivity index using the built-in IPA algorithm. The probability of having a feature observed by random chance was calculated. The statistically significantly determined genes was calculated by a right-tailed Fisher’s exact test with Benjamini–Hochberg multiple-testing correction. The level of significance was set to p < 0.05. Accordingly, the IPA analysis identified the molecular and cellular pathways from the IPA library of canonical pathways that were most significant to the dataset (−log (p value) > 1.5).

**Western blot analysis.** Protein concentrations in EVs and cell lysates were determined with the Pierce® BCA Protein Assay kit (Thermo Fisher Scientific). To assess the enrichment of exosomal markers in tick EVs, vesicles were purified from uninfected or infected tick cell lines, salivary glands, and I. ricinus saliva, as described above. Proteins were electrophoresed in 4–15% Mini-PROTEAN® TGX™ precast gels (Bio-Rad) for 1.5 h at 100 V and transferred to 0.2 µm PVDF membranes (Trans-blot Turbo® transfer pack; Bio-Rad) for 30 min in a TransBlot® Turbo® transfer system (Bio-Rad). Membranes were blotted in 5% dry-milk in PBS for 1 h at RT. Membranes were incubated with anti-TSG101 (1:500 dilution; System Biosciences EXOAB-TSG101-1), −CD63 (1:500 dilution; System Biosciences EXOAB-CD63A-1), −ALIX (1:500 dilution; System Biosciences EXOAB-ALIX1-1), −SL2 (1:500 dilution), −Calnexin (1:10,000 dilution; Millipore-Sigma AB2301), −GFPT1 (1 ng/µl; Proteintech 14132-1-AP), −phospho-PKCα (1:500 dilution; Cell Signaling 9407), −phospho-Syk (1:500 dilution; Cell Signaling 4075), −phospho-ERK (1:500 dilution; Cell Signaling 9103), −phospho-AKT (1:500 dilution; Cell Signaling 9272), −phospho-p38 (1:500 dilution; Cell Signaling 9258), −phospho-JNK (1:500 dilution; Cell Signaling 9251), −phospho-ERK (1:500 dilution; Cell Signaling 9251) and −phospho-JNK (1:500 dilution; Cell Signaling 9251). Proteins were run in western blots.
The DNPH and non-DNPH (control)-treated membranes were blotted in 5% dry-milk in PBS-T for 1 h at RT. Carboxylated proteins were detected with rabbit anti-DNP antibodies (1:1000) for 2 h at RT and with anti-Rabbit-HRP-conjugated antibodies (1:10000) for 2 h at RT.

ELISA. We purified EVs from 4 x 10⁷ uninfected and A. phagocytophilum-infected iSE6 cells. Protein carbonylation was confirmed using the OxiSelect™ Protein Carbonyl ELISA kit (Cell Biolabs STA-310). Protein concentrations were measured with the Pierce BCA Protein Assay Kit. In all, 1 µg of protein per well was loaded into a 96-well plate and allowed to bind overnight at 4 °C. Wells were washed three times with 1x PBS and 1x DNPH working solution, incubating the wells for 45 min at RT. Wells were washed five times with 1:1 ethanol/PBS and two times with 1x PBS. Wells were then blocked for 2 h at RT and washed, following the manufacturer’s recommendations. Anti-DNP antibodies were added to each well and the plate was incubated for 1 h at RT under constant shaking. After three washes, HRP-conjugated antibodies were added to each well and incubated for 1 h at RT. Wells were washed five times and protein carbonylation was detected with 1x substrate solution. The reaction was blocked by adding 100 µl of the stop solution and absorbance was read in an iMark microplate reader at 450 nm (Bio-Rad).

PGD and Nrf2 western blots. Vessel blots were probed with anti-Nrf2 (GeneTex GTX5732; 1:1000) and anti-PGD (Thermo Fisher Scientific PA5-27486; 1:1000) polyclonal antibodies. Bands were stained as described above.

Macrophage differentiation. Bone marrow-derived macrophages (BMDMs) were generated from C57BL/6J mice. In brief, mice were euthanized using CO2 and femurs were removed. Femurs were crushed to liberate. Bone marrow was flushed by injecting buffer containing 10% FBS (Gemini Bio-Products), 1x penicillin/streptomycin (Corning), and 1x amphotericin B (Gibco) into one end of the femur with a 25G needle. Cells were seeded onto 12-well plates at 5 x 10⁵ cells per well at 50 g/ml concentration on days 0, 2, and 4 after seeding. Medium was changed on day 4. Cells were incubated at 37 °C 5% CO2 for 6 days to allow macrophage differentiation. The cell line was used for the experiments. Each experiment was repeated at least three times with similar results. Some of the results are shown in Fig. 1A and Supplementary Fig. 1.

Human. Human macrophages were differentiated from peripheral blood mononuclear cells (PBMCs) as described in Noel et al. In brief, human blood was obtained from healthy adult volunteers. PBMCs were purified by 10 min of EDTA-treated human blood using Ficoll-Paque PREMIUM density 1.073 g/ml (GE Healthcare). Contaminating red blood cells were lysed with ACK lysis buffer for 5 min at room temperature (RT). Monocytes were enriched by negative selection using the human Pan Monocyte Isolation Kit (MiltenyiBiotec) and LS Columns (MiltenyiBiotec). Monocytes were resuspended in RPMI supplemented with 10% FBS (Gemini Bio-Products), 1x penicillin/streptomycin (Corning), and 1x amphotericin B (Gibco) into one end of the femur with a 25G needle. Cells were seeded onto 96 mm Petri dish plates and incubated at 37 °C 5% CO2. Additional differential medium was added to cells on day 3 after seeding. Cells were incubated for 7 days until completely differentiated.

Human. Human macrophages were differentiated from peripheral blood mononuclear cells (PBMCs) as described in Noel et al. In brief, human blood was obtained from healthy adult volunteers. PBMCs were purified by 10 min of EDTA-treated human blood using Ficoll-Paque PREMIUM density 1.073 g/ml (GE Healthcare). Contaminating red blood cells were lysed with ACK lysis buffer for 5 min at room temperature (RT). Monocytes were enriched by negative selection using the human Pan Monocyte Isolation Kit (MiltenyiBiotec) and LS Columns (MiltenyiBiotec). Monocytes were resuspended in RPMI supplemented with 10% FBS (Gemini Bio-Products), 15 µM 2-Mercaptoethanol (Gibco), 1 mM Sodium Pyruvate (Gibco), 1x MEM non-essential amino acids (Gibco), and 1x penicillin/streptomycin (Corning). Cells were counted using Trypsin blue stain (0.4%; Thermo Fisher Scientific) in a TC20™ Automated cell counter (Bio-Rad) and 2 x 10⁵ monocytes were seeded into six-well plates (Sigma). Human recombinant Macrophage Colony-Stimulating Factor (M-CSF; Biolegend) was added to each well at 50 ng/ml final concentration on days 0, 2, and 4 after seeding. Medium was changed on day 4. Cells were incubated at 37 °C 5% CO2 for 6 days to allow differentiation into M0 macrophages.

EV labeling. PKH26 staining. Tick cell EVs were stained as described in van der Vlist et al. Staining solution was prepared by mixing 1.5 µl of PKH26 with 100 µl diluent C in a microfuge tube. PKH26 is a red fluorescent dye with long aliphatic tails. In Fig. 2c, g, PKH26 was artificially transformed to an orange color to be visualized by color-blind readers. EVs (5 µg) were resuspended in 20 µl of 0.2% BSA in PBS precleared of protein aggregates by overnight ultracentrifugation at 100,000 x g at 4 °C and diluted into 80 µl diluent C. EVs were transferred to the staining mix, mixed by pipetting, and incubated at RT for 3 min. The labeling was stopped by adding 40 µl of red dye free IMDM. Protein aggregates and extra dye were separated from the labeled EVs with a 300-kDa Vivaspin500 filter (Sartorius). EVs were washed three times with IMDM and resuspended in phenol red-free IMDM.

Dio staining. Tick cell EVs were labeled by adding 2.5-5 µl of Vibrant Dio cell-labeling solution (Invitrogen) to 480 µg of EVs in 500 µl of EV-free IMDM medium in a microfuge tube. Dio staining was incubated for 20 min at 37 °C. EVs were washed three times with EV-free IMDM and resuspended in 100 µl of EV-free IMDM.

EV-macrophage interactions. Confocal microscopy. BMDM and human macrophages were isolated as described above. Cells (3 x 10⁵) were seeded onto 35 mm glass bottom dishes (MatTek Corporation) and allowed to attach overnight. Medium was replaced using phenol red-free, EVs-free IMDM medium and 5 µM Cytochalasin D (Santa Cruz Biotechnology). Cells were incubated for 30 min at 37 °C under 5% CO2, PKH26-labeled EVs (25 µg/MatTek) were added after incubation with Cytochalasin D and cells were incubated for 4-5 h at 37 °C under 5% CO2 to allow binding. Control cells did not receive EVs. Cell plasma membrane was labeled with CellMask™ Green plasma membrane stain (1:1000; Invitrogen) at 37 °C for 5 min. The medium was replaced with phenol red-free, EV-free IMDM medium containing 5 µM Cytochalasin D. Z-stacked images were acquired with a LSM 5 Live DUOScan laser scanning microscope (Zeiss). Composites were constructed using ImageJ (NIH) Z projection (maximum intensity).

Live-cell confocal microscopy. BMDMs were seeded and treated as described above. Cells were labeled with CellMask™ Green plasma membrane stain (Invitrogen). Medium was replaced with EVs-free IMDM medium containing 5 µM Cytochalasin D. Cells were placed in an external unit and kept at 37 °C under 5% CO2. Z-stacked images were acquired every 5 min for 2 h with a LSM 5 Live DUOScan laser scanning microscope (Zeiss). Twenty-five µg of PKH26-labeled EVs were added after time 0 for the positive control. No EVs were added to the negative controls. Z-projections (maximum intensity) of each channel and videos of the Z projections were constructed in ImageJ at 2 frames-per-second (fps). Arrows were added with Windows Video Maker (Windows).

Flow cytometry. BMDMs and human macrophages were differentiated, as described above. Differentiated macrophages were resuspended in EV-free IMDM medium and aliquoted into microfuge tubes. Cells (5 x 10⁵) were incubated with 40 µg of Dio-labeled EVs, 40 µg of unlabeled EVs, a combination of 20 µg of Dio-labeled and 20 µg of unlabeled EVs, or no EVs for 45 min at 37 °C under 5% CO2. Cells were centrifuged at 300 x g for 10 min to eliminate unbound EVs. Macrophages were resuspended in 250 µl of EV-free IMDM medium and labeled with either APC-labeled anti-mouse F4/80 antibodies (Invitrogen MF48005; 1:100) or APC/ CD14™-labeled anti-human CD11b (Biolegend 301341; 1:100) and APC-human CD14™-labeled anti-human CD14 (eBioscience 17-0149-41; 1:100) for 30 min at RT. Three replicates were left untreated as negative controls. Excess antibody was eliminated by washing for 10 min and resuspending cells in 1% paraformaldehyde in PBS. Cell fluorescent labeling was analyzed using an LSR II (BD Biosciences). Data was analyzed with FCS Express 6 Flow Research Edition (De Novo Software). Experiments were performed in the Flow and Mass Cytometry Core Facility of the University of Maryland School of Medicine Center for Innovative Biomedical Resources (CIBR).

RNAi silencing. Tick homologs of proteins involved in exosome biogenesis in human cells (Supplementary Data 1) were determined using PSI-BLAST. These homologs were used to re-construct the exosome biogenesis pathway in the I. scapularis genome. Small interfering RNAs (siRNAs) and scramble RNAs (scRNAs) were designed for two v-SNAREs, synaptobrevin 2 (vamp2) and vamp3 (NCBI reference in Supplementary Data 6). Silencing (Si)RNAs were designed using BLOCK-iT™ RNAi designer (http://rnaidesigner.thermoscientific.com/raeinexpress/). Scramble (Sc) RNA was designed using GenScript (https://www.genscript.com/tools/create-scrambled-sequence). Both Si and ScRNAs were blasted against the I. scapularis genome to avoid off-target effects and synthesized according to the Silencer® siRNA construction kit (Thermo Fisher Scientific). Primers are described in Supplementary Data 6.

In vitro silencing. iSE6 (~2 x 10⁵) cells were plated in 25 cm² flasks and transfected with 30 µg of SiRNA or ScRNA against synaptobrevin 2 or vamp3 diluted in 50 µl of normal L15C300 medium, 25 µl of Lipofectamine 2000 (Invitrogen) and 2 ml of TRIzol™ reagent (Thermo Fisher Scientific) and stored at ~80 °C until RNA isolation. RNA was isolated from 600 µl of the TRIzol suspension using PureLink™
RNA mini kit (Thermo Fisher Scientific). RNA was measured using a NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific). cDNA was synthesized from 150 to 400 ng of RNA using Verso cDNA synthesis kit (Thermo Scientific).

In vivo silencing. Unfed nymphs were microinjected with 50-80 ng of Si or ScRNA against synaptobrevin 2 or vamp33 using a Nanoject III (Drummond Scientific Company). Ticks recovered overnight at 25 °C with saturated humidity. Live ticks were placed on mice and allowed to feed for 3 days. Ticks were stored at −80 °C for RNA purification. To purify mRNA, ticks were fast-frozen in liquid nitrogen and crushed with small plastic pestles. TRIzol™ reagent (200 µl) was added to the crushed tick and RNA was purified using the PureLink™ RNA mini kit. cDNA was synthesized from 50 to 200 ng (5–10 µl) of RNA using Verso cDNA synthesis kit (Thermo Scientific).

Gene expression. Primers to determine the expression levels of synaptobrevin 2, vamp33, and I. scapularis actin (Supplementary Data 6) were designed using Geneious R10 (Biomatters Inc.) and checked for specificity with Primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Standard curves were produced using the PCR4 plasmid. To determine copy numbers, the mass of the plasmid with each amplicon was determined using the Sequence Manipulation suite DNA molecular weight (http://www.bioinformatics.org/sms2/dna_mw.html) and the copy number calculator (http://scienceprimer.com/copy-number-calculator-for-realtime-pcr/). Levels of gene expression were measured by absolute quantification using Power SYBR™ Green PCR master mix (Thermo Fisher Scientific) and normalized to the expression of actin. Amplifications were done using the following conditions: a denaturation cycle of 10 mins at 95 °C, followed by 40 cycles of denaturation for 15 s at 95 °C, amplification at two different annealing temperatures (synaptobrevin and vamp33: 51 °C; tick actin: 57 °C) for 1 min. Primers were used at a final concentration of 400 µM each and 2 µl of cDNA were used. The efficiency and specificity of the products was determined by single peaks in the melting curves. Quantitate PCR was collected in the Applied Bio-systems 7500 Fast Real-Time PCR System through the 7500 Software v2.3.

Weight measurements. Ticks were microinjected with silencing RNA (vamp33 si or synap2 si) or scramble RNA (vamp33 sc and synap2 sc). Surviving ticks were placed on C57BL/6j (WT), FVB/N jax, FVB/N Tac, or knockout mice to feed for 3 days. Ticks were recovered from a water trap (fully engorged) or were removed with forceps (partially engorged). The percentage of attachment was calculated by 40 cycles of denaturation for 15 s at 95 °C, amplification using the following conditions: a denaturation cycle of 10 mins at 95 °C, followed by a fluorescence readout for 1 min. The threshold value was set to 0.1. Primers were used at a final concentration of 400 µM each and 2 µl of cDNA were used. The efficiency and specificity of the products was determined by single peaks in the melting curves. Quantitate PCR was collected in the Applied Bio-systems 7500 Fast Real-Time PCR System through the 7500 Software v2.3.

Inflammation at the bite site. To determine the degree of inflammation at the bite site, skin samples were taken from mice infected with I. scapularis nymphs at day 3. Control samples consisted of skin from non-infected mice matched to the infected groups where ticks had been the infected mice. Biopsy punches of 2-mm were taken, and skin was trimmed to the proximity of the tick mouthparts. Skin samples were placed in 10% Neutral Buffered Formalin (v/v) for 2–3 weeks before paraffin embedding. Tissue was vertically cut, and sections were hematoxylin and eosin (H&E) stained. Pictures were taken with a Leica DFC495 microscope (Leica). The thickness of the epidermis was measured using ImageJ.

Chemokine and cytokines. I. scapularis nymphs were microinjected with vamp33 si or vamp33 sc RNAs. Microinjected ticks were placed on C57BL/6j (WT) mice and allowed to feed for 3 days before the skin biopsy. The bite site was marked with a permanent marker and ticks were removed with forces. Skin biopsies were taken from the bite site using a 3-mm Integra Miltex disposable biopsy punch (Integra). The weight of the punch biopsies was measured using a Pioneer analytical balance (OHAUS) and the differences in engorgement were evaluated.

Cell populations affected during tick feeding. I. scapularis nymphs fed on C57BL/6j mice. At the 3rd day of feeding, mice were euthanized with CO2 and the site where ticks were feeding was shaved. A 10-mm skin punch biopsy was taken while ticks were still attached. Skin samples from control mice were taken from matching locations. Single cell suspensions were prepared from each skin sample. Briefly, skin samples were cut into small pieces with sterile surgical scissors and placed into 14 ml FALCON® polypropylene round-bottom tubes containing 3 ml digestion buffer consisting of 90% RPMI-1640 (Quality Biological), 10% L-β-mercaptoethanol (L-β-ME) (Thermo Fisher Scientific), Roche Diagnostics, 0.1% protease inhibitor cocktail (Thermo Scientific), and 0.01% aprotinin. The percentage of attachment was calculated from the total number of ticks recovered divided by the total number of ticks microinjected. The weight of the ticks was measured using a Pioneer® analytical balance (OHAUS) and the differences in engorgement were evaluated.

Skin immune cells population. Skin cell populations affected during tick feeding. I. scapularis nymphs fed on C57BL/6j mice. At the 3rd day of feeding, mice were euthanized with CO2 and the site where ticks were feeding was shaved. A 10-mm skin punch biopsy was taken while ticks were still attached. Skin samples from control mice were taken from matching locations. Single cell suspensions were prepared from each skin sample. Briefly, skin samples were cut into small pieces with sterile surgical scissors and placed into 14 ml FALCON® polypropylene round-bottom tubes containing 3 ml digestion buffer consisting of 90% RPMI-1640 (Quality Biological), 10% L-β-mercaptoethanol (L-β-ME) (Thermo Fisher Scientific), Roche Diagnostics, 0.1% protease inhibitor cocktail (Thermo Scientific), and 0.01% aprotinin. The percentage of attachment was calculated from the total number of ticks recovered divided by the total number of ticks microinjected. The weight of the ticks was measured using a Pioneer® analytical balance (OHAUS) and the differences in engorgement were evaluated.
Genetic ablation of γδ and γε T Cells. Ticks were microinjected with vamp33 si or vamp33 sc and placed on C57BL/6(j) (WT), ccr2−/−, ccr6−/−, tcrβ−/−, tcrγδ−/−, FVBN/J, and FVB/N Tac mice. Ticks fed for 3 days and their weight was measured.

**FTY720 treatment in mice.** C57BL/6 (WT) mice were injected intraperitoneally (i.p.) with FTY720 (Sigma-Aldrich), 1 mg/kg in 100 µL sterile water on days 1 and 7 and on the morning of day 0 prior to tick challenge.

Ticks were microinjected with vamp33 si or vamp33 sc. Ticks fed on FTY720-injected PBS-injected mice for 3 days. Mice were euthanized on the third day. Ticks were weighed. In all, 10 mm skin punch biopsies and draining lymph nodes were obtained from euthanized mice. FTY720 treatment was confirmed through FACS measurement of lymphocytes in the skin and draining lymph nodes. Skin and lymph node samples were labeled using APC-Cy7 anti-CD4 (Miltenyi Biotec 130-118-568; 3 ng/µl), PerCP-Cy5.5 anti-CCR6 (Miltenyi Biotec 130-116-655; 5 ng/µl), and PECy7 anti-CCR7 (Miltenyi Biotec 130-116-530; 3 ng/µl). Live and dead cells were discriminated using propidium iodide solution (Miltenyi Biotec). Cell populations were measured with a MACSQuant flow cytometer and analysis was performed using FlowJo software v 10.6.1.

**Intracellular bacterial infection.** A. phagocytophilum model. Female C57BL/6 mice were shaved in three different spots on the back. Cell-free A. phagocytophilum (1 × 10⁶ bacteria/injection) re-suspended in 50 µl of PBS were intradermally (i.d.) injected into these spots in the presence or absence of 4 × 10⁷ I. scapularis nymphal salivary EVs. Infections progressed for 48 h and animals were euthanized using CO₂. Blood samples were taken by heart puncture and divided into two specimens: half of the blood in a serum collection tube and the other half in EDTA Microvette 200 µl tubes (Sarstedt Ag). Skin biopsies were taken from each injection site, and spleens and livers were dissected. Skin and spleen samples were placed at −80°C. DNA was extracted from skin samples, using the DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer’s recommendations. DNA was eluted in 100 µl of elution buffer. DNA concentration was determined by standard spectrophotometrical measurements. DNA quantity was verified by amplification of a single copy gene γβδ (16S). Gene expression was normalized by the housekeeping gene Porphyromonas gingivalis Actin. DNA was further used for the isolation of DNA repletion (e.g., day 0, week 2, week 4, week 6, etc.), ticks were surface-sterilized by exposing them for 2 min to 70% ethanol for 5 min. Following 2 h of incubation, the number of conscious per plate was counted and CFU/ml (mouse blood) or CFU/ml was calculated.

*Ethics statement.* Blood collections were performed on healthy volunteers who provided informed consent. The protocol was approved by the Institutional Review Board (IRB# HP-00040025) of the University of Maryland School of Medicine and comply with the 21 CFR part 50. I. ricinus feeding experiments were performed in accordance with the Animal Protection Law of the Czech Republic (157 Act No. 240/1991) and with the approval of the Academy of Sciences of the Czech Republic (approval no. 162/2010). All mouse experiments were carried out under the guidelines approved by the Institutional Biosafety Committee (IBIC#0000002247) and Animal Care and Use Committees (IACUC#0216015 and IACUC#0101012) at the University of Maryland School of Medicine and (IACUC#1086627 and IACUC#1086665) at the University of Toledo College of Medicine and Life Sciences. The Office of Human Subjects Research at the National Institutes of Health (NIH) guidelines (Office of Laboratory Animal Welfare (OLAW) #A3200-01, A323-01, and A3270-1). Calf experiments were done in accordance with the guidelines approved by the IACUC at the University of Idaho (IACUC# 2017-39).

**Tick acquisition and transmission of *F. tularensis*.** One day prior to tick challenge, C57/HeN mice were anesthetized with a ketamine-xylazine sedative, an area ~2.5 cm in diameter between the shoulder blades was shaved with surgical clippers, and plastic chambers (top portion of 15-ml conical tubes) were adhered to shaved skin using Kamar adhesive. Mice were individually housed to prevent cannibalism or tick biting of cages. The next day (day -5), mice were anesthetized, nymphal *D. variabilis* ticks were placed in each chamber, chambers were closed and ticks were attached by polymer foam to the side of each chamber using a ne-mesh polyester fabric. Double-sided tape was adhered to the inside upper rim of each cage bottom and cages were placed on tick mats to prevent loss of any escaped ticks.

Three days after tick placement (day -2), mice were anesthetized, and intradermally injected with *L. monocytogenes* intracutaneous inoculum, which was plated in quadruplicate on MDH to confirm CFUs. Approximately 48 h after infection (day 0), mice were anesthetized, replete ticks were collected, and blood was harvested from infected mice by cardiac puncture for serial dilution in PBS and plating. To determine bacterial numbers in ticks at various time points after repletion (e.g., day 0, week 2, week 4, week 6, etc.), ticks were first surface-sterilized by placing into 30% H₂O₂ for five seconds, 70% ethanol for 5 s, washed with molecular grade water (Corning) for 5 s. Then, homogenized in RNase-free disposable pellet pestles pestle tubes (Fisher) containing 200 µl of sterile PBS. Tick homogenates were serially diluted in PBS and plated onto MHA containing 100 mg/L cycloheximide, 80,000 U/L polymyxin B, and 2.5 mg/L amphotericin B. Following 72 h of incubation, the number of colonies per plate was counted and CFU/ml (mouse blood) or CFU/ml was calculated.

Infection studies were performed essentially as described above, with the following modifications: LVS-infected adult ticks (14 weeks after their nymphal blood meal) were individually placed into chambers and allowed to feed to repletion on naive mice. Replete adult ticks were collected 8 days after tick placement and were either immediately homogenized for bacterial enumeration (day 8) or homogenized for bacterial enumeration when associated mice were moribund (days 14–18). To quantify bacterial burdens in mouse blood and tissues during infection studies, mouse blood/tissues were either collected on day 8 (moribund animals) or when mice were moribund (days 14–18). Mice were anesthetized, blood was collected by cardiac puncture, and mice were cervically dislocated, skin from the tick attachment site was harvested using a 8 mm biopsy punch (Accuderm), and lungs, livers, and spleens were aseptically harvested and transferred to sterile Whirlpack bags. Samples were homogenized, 25 µl of PBS/mg of tissue was added to each tube, serially diluted, and dilutions were plated onto media.

**Statistical analysis.** Statistical significance of each experiment was assessed with the unpaired one- or two-tailed Student’s t-test. One-way or Two-way ANOVA, p < 0.05 was performed as an Excel test for multiple comparisons and Two-way ANOVA followed by Fisher’s least significance difference statistical tests were also used whenever appropriate. Survival curves were analyzed with the Kaplan–Meier followed by the
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Log-rank (Mantel-Cox) test. We used GraphPad PRISM® (GraphPad Software version 9.1.0) for all statistical analyses. Outliers were detected by a Graphpad Quickcalc program (https://www.graphpad.com/quickcalc/Graphbs1.cfm).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE99 partner repository with the dataset identifier PXD018779 under the project name “Label free proteomics profiling of nanovecicle isolated from cultured salivary glands isolated from partially fed adult female Ixodes scapularis” Project https://doi.org/10.6019/PXD018779. All other data are available upon reasonable request. Raw data from all experiments are available as Source Data file. Source data are provided with this paper.
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Author contributions
A.S.O.C. performed the research with contributions from X.W., L.M., N.K.A., H.L.H., D.K.S., B.G.T., E.E.M.C., S.L.F., L.R.B., P.S., A.D.B., K.M., L.L., A.J.O., K.L.M., B.E.H., D.E.S., and M.K. Reagents and advice were provided by C.B.M., J.G.V., M.F.P., U.P., S.M.J., J.F.H., L.M., G.A.S., E.M.B., M.L.L., and L.S. The project was developed by A.S.O.C. and supervised by J.H.F.P. with input from M.K., E.M.B., and L.S.M. The paper was written by A.S.O.C. and J.H.F.P. with help from the other authors.

Competing interests
L.S.M. is a full-time employee of Janssen Pharmaceuticals and holds Johnson & Johnson stock. L.S.M. performed all work at his prior affiliation at Johns Hopkins University School of Medicine and he has received prior grant support from AstraZeneca, Pfizer, Boehringer Ingelheim, Regeneron Pharmaceuticals, and Moderna Therapeutics. L.S.M. was also a paid consultant for Armarill, AstraZeneca, Moderna Therapeutics and Janssen Research and Development. L.S.M. was on the scientific advisory board of Integrated Biotherapeutics and is a shareholder of Noveome Biotherapeutics. All of these aforementioned companies are developing therapeutics against infections and/or inflammatory conditions. The remaining authors declare not competing interest.

Additional information
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Correspondence and requests for materials should be addressed to J.H.F.P.

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