BACTERIAL COMPARISONS: HOW ANAPLASMA PHAGOCYTOPHILUM AND BORRELIA BURGDORFERI EFFECT THE SERETION OF VESICLES IN THEIR VECTOR, AND THE IMMUNE RESPONSE OF THEIR HOST

A Thesis

by

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ABSTRACT

Lyme disease and Human Granulocytic Anaplasmosis (HGA) are the two most reported tickborne diseases in the US. Nevertheless, what happens locally at the skin during transmission of *Anaplasma phagocytophilum*, the causative agent of HGA, is poorly defined. In this study, RNAseq analysis of the bite sites of *A. phagocytophilum* infected ticks compared uninfected ticks indicate that many immune related genes are upregulated during *A. phagocytophilum* transmission, particularly those related to the IFN-γ, NF-κB and JAK/STAT signaling pathways. Multiple cytokines and chemokines were also upregulated. The production of extracellular vesicles from *A. phagocytophilum* and *B. burgdorferi* infected tick midguts and salivary glands was observed, with *A. phagocytophilum* infection resulting in extracellular vesicles 50-150 nm in diameter being significantly reduced in both organs. *B. burgdorferi* infection reduced midgut vesicles but produced no significant change in salivary glands. Overall, these results indicate that the response of the host and the vector to different pathogens is unique.

DEDICATION

I dedicate this dissertation to my Mother and Father: Caryn and Ron Underwood. They are two of the greatest people in the world. Without them I most certainly wouldn't be where I am today as their dedication to my success has allowed me to achieve goals I would have thought impossible. To my Mother, I love you. Your kindness has helped me through everything. To my Father, thank you for pushing me that little bit further every time I needed it. I wish to be a great man and father like you one day.

"Imagination is more important than knowledge. For knowledge is limited to all we now know and understand, while imagination embraces the entire world, and all there ever will be to know and understand."

-Albert Einstein-

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Contributors

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Gene ontology and expression values were calculated by GeneWiz. Initial *A. phagocytophilum* infections for gene expression analysis were completed with the aid of Dr. Adela Oliva Chavez and Cross Chambers. *Borrelia burgdoferi* infection, vesicle, and cytokine measurements were completed by the student independently.

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CHAPTER I

1. Ticks

Ticks are a group of obligate blood-feeding arthropods consisting of three families: the Argasidae (soft ticks), Ixodidae (hard ticks), and the single species of Nuttallielidae [1, 2]. These organisms are well-known vectors of disease, with members of the family Ixodidae being competent vectors of bacteria, viruses, and protists [3]. Unlike Argasids, who feed for relatively short periods of minutes to hours, hard ticks remain attached to their host for days to weeks at a time [4]. Feeding is done through laceration of the host dermis by the chelicerae, forming a feeding pool where the hypostome is then inserted [5]. This invasive and destructive feeding behavior results in the stimulation of inflammatory and immune responses in the skin. To avoid rejection by the host, ticks have evolved the ability to dampen their host's immune response. This immune-modulation occurs through a complex process involving a cocktail of salivary effectors, resulting in reduced inflammation, increased blood flow, and suppression of immune related cells at the bite site [6-9]. Recent studies have shown that these salivary effectors are secreted within extracellular vesicles [10-13], which will be discussed in more detail below.

1.1 *Ixodes scapularis*

Ixodes scapularis belongs to the Ixodidae family. It is a three-host tick, detaching from its host after each feeding for molting or reproduction, with a life cycle of 2-4 years [14]. The immature ticks feed on rodents, birds, and other small to moderate sized mammals in their environment [15] (Figure 1). Adult ticks feed on medium to large sized mammals, with white-tailed deer (*Odocoileus virginianus*) being the primary host [16]. This association is so well known it is noted in the common name of *I. scapularis*: the blacklegged deer tick. This life history places humans as incidental hosts of *I. scapularis* and a deadend host for its associated pathogens. Thus, any feeding that takes place on humans is nonessential to the maintenance of tick populations and the transmission cycle of tick-borne pathogens.

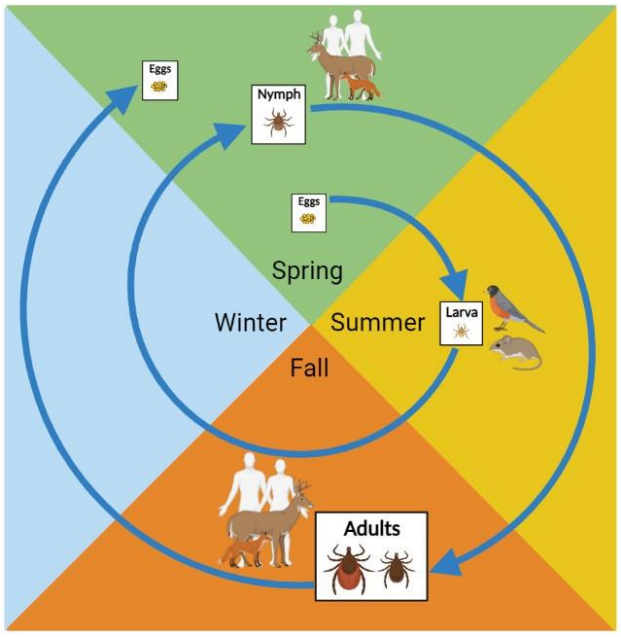

Figure 1. Life cycle *I. scapularis.* Modified from [13]. Created with BioRender.com.

1.2 *I. scapularis* range

I. scapularis was originally described in the United States from an area near Cape Cod, Massachusetts in the 1920s [17]. Since then, it has been described across a broad geographic range across the eastern areas of the country. Common in the Eastern United States, *I. scapularis* has been experiencing a range expansion over the last 20 years. The number of counties

considered to have established *I. scapularis* populations has more than doubled. Counts from 2016 place *I. scapularis* in 1420 of the 3110 counties in the contiguous United States [18]. Although unconfirmed, models of environmental suitability imply even more counties are likely to harbor populations of *I. scapularis* [19, 20]. A similar trend has been observed in the distribution of *B. burgdorferi* and in the number of instances of Lyme disease [18, 21, 22], with the number of confirmed cases increasing from approximately 10,000 to 27,000 from 2008 to 2015. Thus, the impact of *I. scapularis* and *I. scapularis*-borne diseases in the US is increasing.

2. Tick-borne Pathogens

Annually, approximately 50,000 cases of locally acquired vector borne diseases are reported in the United States. Of these, 95% are tick-borne pathogens with greater than 70% of these cases being Lyme disease [23]. Lyme disease is predominately caused by the bacterium *Borrelia burgdorferi*, with a small number of cases caused by *Borrelia mayonii* [24]. Seven tick-borne pathogens affecting humans have been shown to be transmitted by *I. scapularis*. This includes *Anaplasma phagocytophilum*, *B. burgdorferi*, *Babesia microti*, Powassan virus, and the recently discovered *B. mayonii*, *Borrelia miyamotoi*, and *Erlichia muris* sp. *eauclarensis*, [21, 25] (Figure 2). This rapid expansion in the number of tick-borne pathogens in due to the relatively recent use of polymerase-chain reaction (PCR) analysis for diagnostic purposes, allowing for an efficient method for correlating microorganisms with human disease [26] (Figure 2). Herein, I will focus on *B. burgdorferi* and *A. phagocytophilum*, which affect the largest number of people in the US.

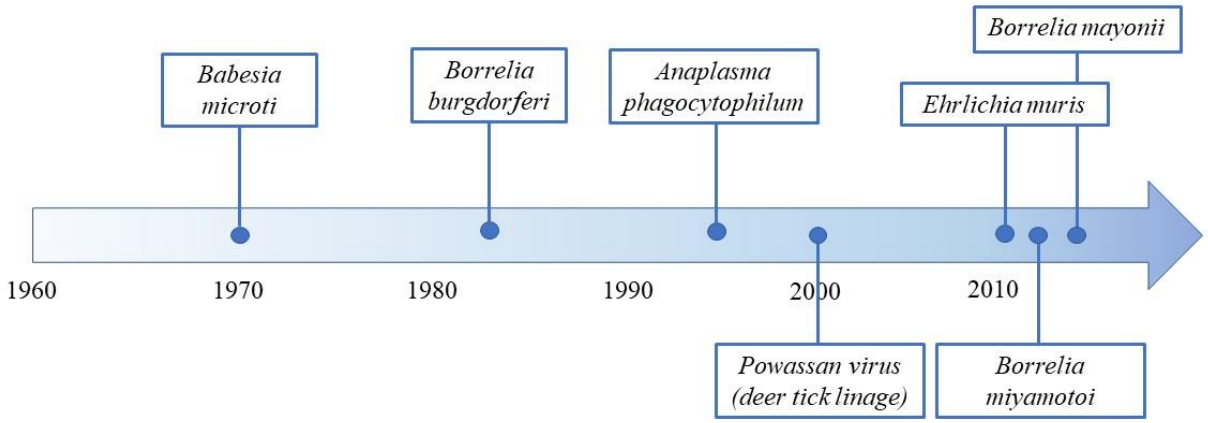

Figure 2. Timeline Showing Discovery of the Seven Human Pathogens Transmitted by *Ixodes scapularis*. Adapted from [27].

2.1 *Borrelia burgdorferi*

Lyme disease, caused by spirochete bacterium *B. burgdorferi*, has multiple symptoms and phases. Initial phases beginning in the skin (with erythema migrans being a key clinical sign in 80% of cases) and other generic flu-like symptoms, such as headaches, fever, and malaise [28]. As the bacteria disseminate into deeper tissues the disease progresses into its chronic phase, effecting the heart, joints, and nervous system. Signs of chronic infection include skin rash and arthritis, with severe cases of chronic disease resulting in cardiac illness and various neurological disorders [3].

B. burgdorferi is a eubacterial organism in the phylum Spirochaetes. Spirochetes are characterized by their wavelike or spiral morphology and the presence of flagella between the outer and inner membranes of the cell. This group of bacteria harbors several other human pathogens, including the causative agents for syphilis (*Treponema pallidum*), leptospirosis (*Leptospira interrogans*), and other *Borrelia spp.* known to cause relapsing fevers [29].

Lyme disease was initially considered a novel type of inflammatory arthritis upon its discovery in 1975 [29]. *B. burgdorferi* was first isolated from the midguts of the black-legged tick, *I. scapularis* by Dr. Willy Burgdorfer in 1982 [30]. *B. burgdorferi* can infect a broad range of hosts, such as mammals and birds. In the United States, *I. scapularis* and *Ixodes pacificus* acquire the spirochete as larvae from a reservoir host (typically a rodent). Due to the feeding done by adult ticks being the last feeding of their life cycle and their large size in relation to nymphs, nymphs are the primary vectors of tick-borne pathogens to humans [31].

2.1.1 *Borrelia burgdorferi* biology and infection

B. burgdorferi has several unique characteristics as an extracellular bacterial pathogen. The first is its minimalistic genome, limited in genes encoding metabolic pathways, virulence, and defense mechanisms. Furthermore, twelve plasmids are associated with *B. burgdorferi* infectivity (though there are many others), and a loss of any one of these plasmids could result in the organism not being able to persist within its host or vector [32-35]. The only complete metabolic pathway that has been identified within the *B. burgdorferi* genome is the glycolytic pathway, which is active while they obtain their source of energy from the host or vector. This bacterium lacks genes that encode the enzymes of the citric acid cycle and for ATP synthesis through oxidative phosphorylation. Thus, it utilizes sugar fermentation into lactic acid and glycolysis for energy production. *B. burgdorferi* is required to scavenge simple molecules like glucose, glycerol, trehalose, and chitobiose (an N-acetyl glucosamine dimer derived from chitin) from its host or vector to fulfill its basic needs for survival [36]. Furthermore, amino acid and lipid synthesis pathways in its genome are either incomplete or absent [37]. *B. burgdorferi* restructures its outer membrane by adding host-derived lipids. The addition of host lipids may serve as a potential immune evasion mechanism [38].

An important part of the *B. burgdorferi* life cycle is a period of reduced activity inside of the tick vector. While in the tick, the bacterium uses the outer surface protein A (OspA) to bind TROSPA, a midgut protein that is expressed during feeding [39, 40]. OspA may also shield the organism from host antibodies present in the blood meal when establishing in the tick. *B. burgdorferi* mutants lacking OspA have difficulty establishing infection in the tick midgut when tick feeding takes place on *B. burgdorferi* immune hosts [39].

The transition from the tick to the host during transmission is largely influenced by the temperature change that occurs in the midgut during feeding (<34C to 37 °C), which stimulates bacterial growth and the alteration of bacterial surface proteins [41, 42]. The change in temperature triggers the alteration of *B. burgdorferi* surface proteins. Outer surface protein C (OspC) is upregulated, while OspA and outer surface protein B (OspB) are downregulated. This switch is essential, as OspC is required for infection [41]. It is thought that while OspC is an antigenic protein, it plays a role in immune evasion from mononuclear phagocytes (macrophages) and induces vascular endothelial growth factor for the dissemination of the organism [43-45]. Shortly after establishment the outer surface protein known as variable lipoprotein surface-exposed protein (VlsE) is produced. This protein is antigenically variable. Antigenic variation of VslE is accomplished through the recombination of sequences located on linear plasmid 28-1 into an expression cassette and is necessary for persistent infection [46], [47]. The exception is during the infection of immunodeficient hosts unable to produce antibodies, where the VlsE protein is unnecessary but OspC is still required to establish infection, as will be discussed later in this chapter [48].

The response regulatory protein 2 (Rrp2)– RNA polymerase, nitrogen-limitation N (RpoN)– RNA polymerase, sigma S (RpoS) and histidine kinase Hexokinase-1 (Hk1)– Ribosomal RNA

processing protein 1 (Rrp1) form part of a two component system that regulates the transcription of OspA, OspB, OspC, and VlsE [49]. The Hk1–Rrp1 regulatory pathway is essential for recognizing tick feeding and persistence within the vector [50, 51]. The expression of the key outer surface proteins associated with *B. burgdorferi* virulence are regulated by the alternative sigma factors RpoN-RpoS [52]. This is done partly through the upregulation of the repressor BosR by the RpoN-RpoS pathway, which results in increased expression in OspC and downregulates OspA through binding of the *ospAB* operon [53] (Figure 3).

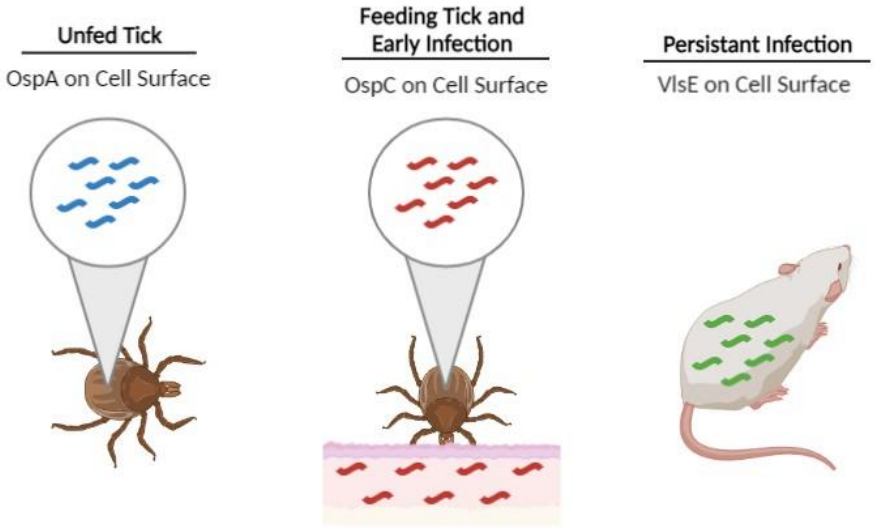

Figure 3. Changes in *Borrelia burgdorferi* outer proteins during transmission cycle. Changes in temperature and other host factors trigger the switch in outer surface protein expression. In the unfed tick, *B. burgdorferi* (here in blue) produces OspA and persists within the tick midgut. After tick attachment and during the bloodmeal intake, *B. burgdorferi* (now in red) produces OspC as it prepares to enter a new host. During infection, *B. burgdorferi* (green) produces VlsE among other proteins to maintain persistent infection. Modified from [31]. Created with BioRender.com.

Following localized establishment within the dermis, *B. burgdorferi* must disseminate from the location of the tick bite into the surrounding tissues. This is done primarily through a

hematogenous route through the blood [54, 55]. *B. burgdorferi* interacts with the vascular endothelium by binding endothelial cells, using decorin-binding protein A (DbpA) in a mechanism termed “transient/tethering”. This is typically done with one end of the bacteria contacting the vessel endothelium as its velocity through the blood slows. This process is thought to reduce dissociation of the bacteria from the endothelium. A second mechanism termed “dragging” involves associating with the endothelium across the length of the bacterium without fully terminating movement. The last mechanism termed “stationary adhesion” is a full termination of movement, by directly binding the endothelium or forming a bridge with fibronectin bridge (Fn) and a host integrin. All of these interactions involve the fibronectinbinding protein BBK32. Another protein able to interact with integrins that is considered important for the transmigration of *B. burgdorferi* is P66 [56-58] (Figure 4).

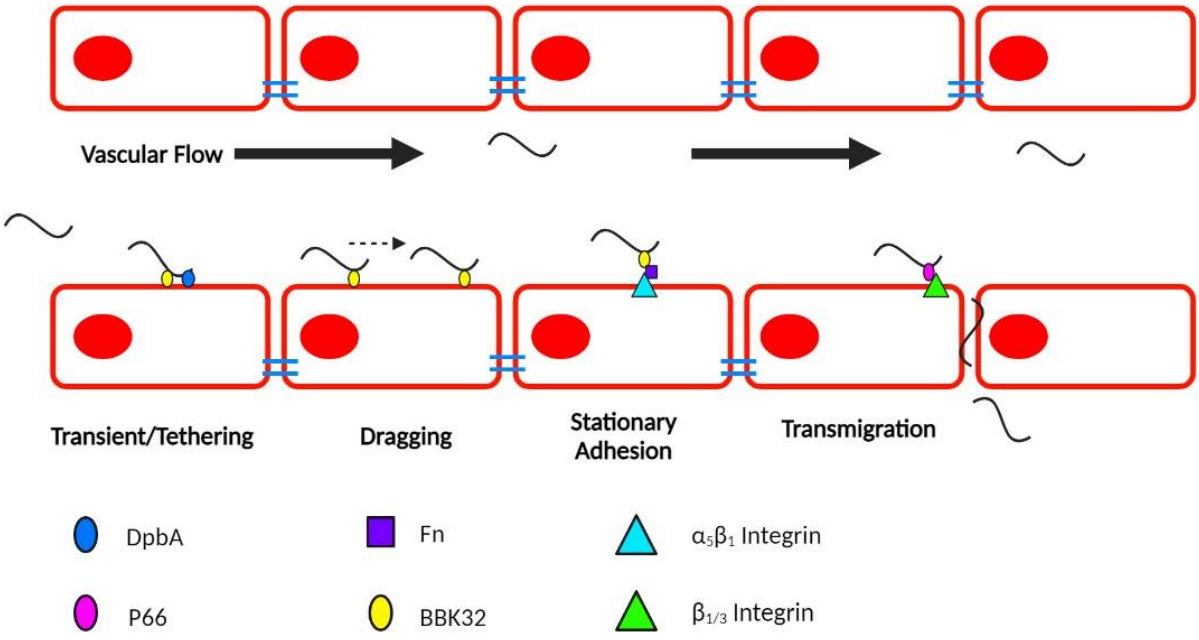

Figure 4. Vascular interactions and transmigration by *B. burgdorferi*. *B. burgdorferi* interacts with host vasculature in three ways: “transient/tethering, dragging, and stationary adhesion”. Decorin-binding protein A (DbpA) functions as a promoter of the transient/tethering interaction with

endothelial cells. All these interactions involve BBK32. Stationary adhesion via BBK32 is accomplished either through direct interactions with the endothelial surface or through the creation of a fibronectin bridge (Fn) with a host integrin. Another protein able to interact with integrins that is considered important for the transmigration of *B. burgdorferi* is P66 Modified from [56]. Created with BioRender.com.

2.1.2 Immune response to *B. burgdorferi* infection

Once in the host, the first obstacle the bacterium must overcome is the host immune response. *B. burgdorferi* is initially recognized by immune cells, such as dendritic cells, when it first enters the skin. Dendritic cells are antigen presenting cells (APCs), meaning that they patrol for invading pathogens, phagocytose them, process, and present their antigens to CD4+ helper T cells [59]. The mammalian response to *B. burgdorferi* is well studied, and the key receptor that appears to be responsible for the detection of the pathogen are toll-like receptors. Studies involving mice devoid to the myeloid differentiation antigen 88 (MyD88), an adapter protein that is required for effective signaling through the Toll-like receptor recognition pathways show increased bacterial numbers [59-61]. An increase in neutrophil recruitment to the bite site has been shown in previous studies, resulting in enhanced control of early *B. burgdorferi* infections [62]. For early infections located in the skin the host immune system employs T cells, activated dendritic cells, and the proinflammatory cytokines IL-6 and IFN-γ [63]. Tick feeding alters these responses, with monocytes showing significantly reduced expression of IL-6, IL-8 and tumor necrosis factor-alpha with ELISA analysis. Meanwhile fibroblasts, another cellular skin component showed enhanced production of IL-6 and IL-8 [64].

During feeding by the tick vector, the host's tissue around the bite site becomes inflamed. Inflammation involves various cell types present in the skin, with the first three hours after infestation resulting in an accumulation of large populations of eosinophils and mast cells near the tick's hypostome. Also observed in the dermis around the bite site is a large population of neutrophils, macrophages, and the secretion of their associated chemokines [65]. After the initial innate response at the bite site, innate immune cells and adaptive immune cells, such as T cells and B cells, interact generating a pathogen or parasite specific immune memory. One such group of cells that bridges innate and adaptive immune responses are γδ T cells. These cells stimulate dendritic cells to produce cytokines and chemokines *in vitro* that are important for the adaptive immune response. Furthermore, γδ T cells are activated and expand in number during *Borrelia* infection, and the absence of γδ T cells results in a significantly reduced expansion of T and B cells and levels of antibodies, cytokines, and chemokines [66]. Other cell types essential in anti-*Borrelia* responses are Th1 and Th17 helper cells. In patients with *B. burgdorferi* erythema migrans, cytokines and chemokines associated with Th17 cells (IL-23, IL-27, IL-25, IL-22, IL17F, IL-21, IL-17A, CCL2, CXCL9, and CXCL10) were significantly increased. Th17 responses in patients with Lyme arthritis correlate with autoimmunity [67]. The Th17 response seen in *B.*
burgdorferi infections seems to arise in part from the recognition of the *B. burgdorferi* neutrophil-activating protein A (NapA) during the establishment of chronic infections [68].

Activated B-cells initially produce IgM antibodies to combat *B. burgdorferi* during initial infection. IgM is an activator of the classical complement pathway responsible for controlling bacteremia. Immunoglobulins will typically undergo class switch recombination to IgG soon after an infection. IgG serves as the main antibody for clearing pathogens. However, during *B.*

burgdorferi infections, serum IgM levels remain high, and the ratio between the two shows no significant change throughout infection [69]. Curiously, *Borrelia burgdorferi* appears to be able to escape antibody recognition. A documented method of immune evasion employed by *B. burgdorferi* is to bind OspC with the tick salivary protein Salp15, which inhibits CD4+ T cell activation, prevents the binding of antibodies, and acts as an antiphagocytic factor. This prevents compliment, antibody, and cellular mediated killing from its host [44, 70-72].

2.2. *Anaplasma phagocytophilum*

Human Granulocytic Anaplasmosis (HGA), caused by the bacterium *Anaplasma phagocytophilum*, is a disease that affects the neutrophils and granulocytes of the immune system [73]. Described first as human granulocytic ehrlichiosis (HGE), the causative pathogen was previously classified in the genus *Ehlichia* with the name *Ehrlichia phagocytophila*. HGA was discovered in six patients in Minnesota and Wisconsin between 1990 and 1993 suffering from an acute febrile illness. In 1996, *I. scapularis* was confirmed to be a vector of *E. phagocytophila* through experimentation, and the white-footed mouse (*Peromyscus leucopus*) was documented as a competent reservoir for the bacterium [74]. The bacterium was later redescribed as *A. phagocytophilum* in 2001 [75].

Clinical signs and symptoms of *A. phagocytophilum* infection typically include fever, malaise, and nausea, occurring 2-3 weeks following exposure from an infected tick [76]. Diagnoses require laboratory tests for confirmation by direct observation of the bacteria in neutrophils and granulocytes via microscopic techniques, through a positive Polymerase Chain Reaction (PCR) of the *16s rRNA* and *p44* genes in blood samples, or by serologic tests [77, 78].

Mortality from Human Granulitic Anaplasmosis is estimated around 1%. Immune compromised individual and those over 40 years of age are at higher risk [79]. Confirmed cases of Human Granulitic Anaplasmosis result in a hospitalization rate of 36%, with 7% of those cases transferred into intensive care [80].

2.2.1 *Anaplasma phagocytophilum* biology

Anaplasma phagocytophilum is an obligate intracellular bacterium, and one of few bacteria capable of infecting phagocytic cells, surviving and replicating within host neutrophils [81]. *A. phagocytophilum* has a byphasic life cycle and takes on two forms: a large reticulate form and a dense-core form with condensed nucleus. Only the dense core cells bind to and invade HL-60 cells, by interacting with P-selectin glycoprotein ligand 1 (PSGL-1) [82-85]. PSGL-1 is the only high affinity ligand for P-selectin on leukocytes and is found in lower levels on B-cells and circulating dendritic cells. This ligand is used for adhesion to the endothelium and activated platelets, facilitating the migration of these immune cells to sites of damage or infection [86]. Neutralization studies have determined that *A. phagocytophilum* binds to host cells through the activity of the major surface proteins Msp2 (*p44*), Asp55, and Asp62, which are potential adhesins [87-89]. Furthermore, the Sialyl Lewis X (sLe^x) is a carbohydrate modification found in PSGL-1 considered to be a critical receptor for *A. phagocytophilum* and is found on all cell types the organism is known to be able to infect: neutrophils, bone marrow progenitor cells, and HL-60 cells [90]. *A. phagocytophilum* binding activates the PSGL-1 signaling pathway in HL-60 cells. The activation of this pathway during bacterial binding results in the tyrosine phosphorylation of the serine/threonine kinase ROCK1, potentially modulating actin reorganization and facilitating bacterial internalization. ROCK1 phosphorylation in infected HL-60 cells is inhibited by

PSGL1-blocking antibodies and small interfering RNA (siRNA) knock-down of a different tyrosine kinase, Syk. Knockdown of either PSGL-1 or Syk impairs infection [91]. Once inside an HL-60 cell, dense-core bacteria transition back to reticulate cells within 12 hours, as these cells initiate replication. *A. phagocytophilum* multiplies within membrane-bound inclusions, known as morulae, in its host cell cytoplasm. As the bacterium divides and proliferates, the morulae expand taking most of the cytoplasm of the host cell. After 36 hours cells are reinfected, with vacuole-enclosed dense-core and reticulate cells present within the same cell [85].

Proteomic analysis has revealed that during infections with *A. phagocytophilum*, HL-60 cells showed an increased expression of proteins involved in cytoskeleton biogenesis, vesicle trafficking, signaling, and energy metabolism [92]. *A. phagocytophilum* manipulates the cellular trafficking platforms in the host cell, known as lipid rafts, to facilitate its entry and infection [93]. This entry also requires the lipid raft associated glycosylphosphatidylinositol (GPI)anchored proteins (GAPs) and Flotillin 1 for infection [93, 94].

As mentioned above, *A. phagocytophilum* has the ability of manipulating its host cells through the secretion of effector proteins. One example is the secretion of AnkA via Type IV secretion system (T4SS). T4SS is a contact-dependent secretion system that spams the envelope of some gram-negative bacteria. They use T4SS to deliver nucleic acids or proteins to neighboring cells [95, 96]. AnkA, a T4SS effector, alters the structure of the hosts chromatin by binding stretches of AT-rich DNA, affecting the transcription of genes involved in antimicrobial responses [97, 98]. *A. phagocytophilum* prevents the fusion of its vacuole with lysosomes, small intracellular vesicles containing digestive enzymes characterized by low pH, by interfering with the vesicular trafficking of its host cell[99, 100]. Morulae also localize with Rab1, a mediator of trafficking from the endoplasmic reticulum to the Golgi apparatus, and Rab4, Rab10, Rab11, Rab14, Rab22,

and Rab35, which are regulators of endocytic recycling and vesicular trafficking [101, 102]. Interestingly, the application of tetracycline, an inhibitor of protein synthesis through the binding of the ribosomal A-site, alters the association of the morulae to Rab proteins [101], indicating that this is an active mechanism likely facilitated by an unknown *A. phagocytophilum* effector. Several *A. phagocytophilum* effectors are known to associate with the membranes of morulae, where they interact with the host proteins, although their exact function is unknown (Figure 5).

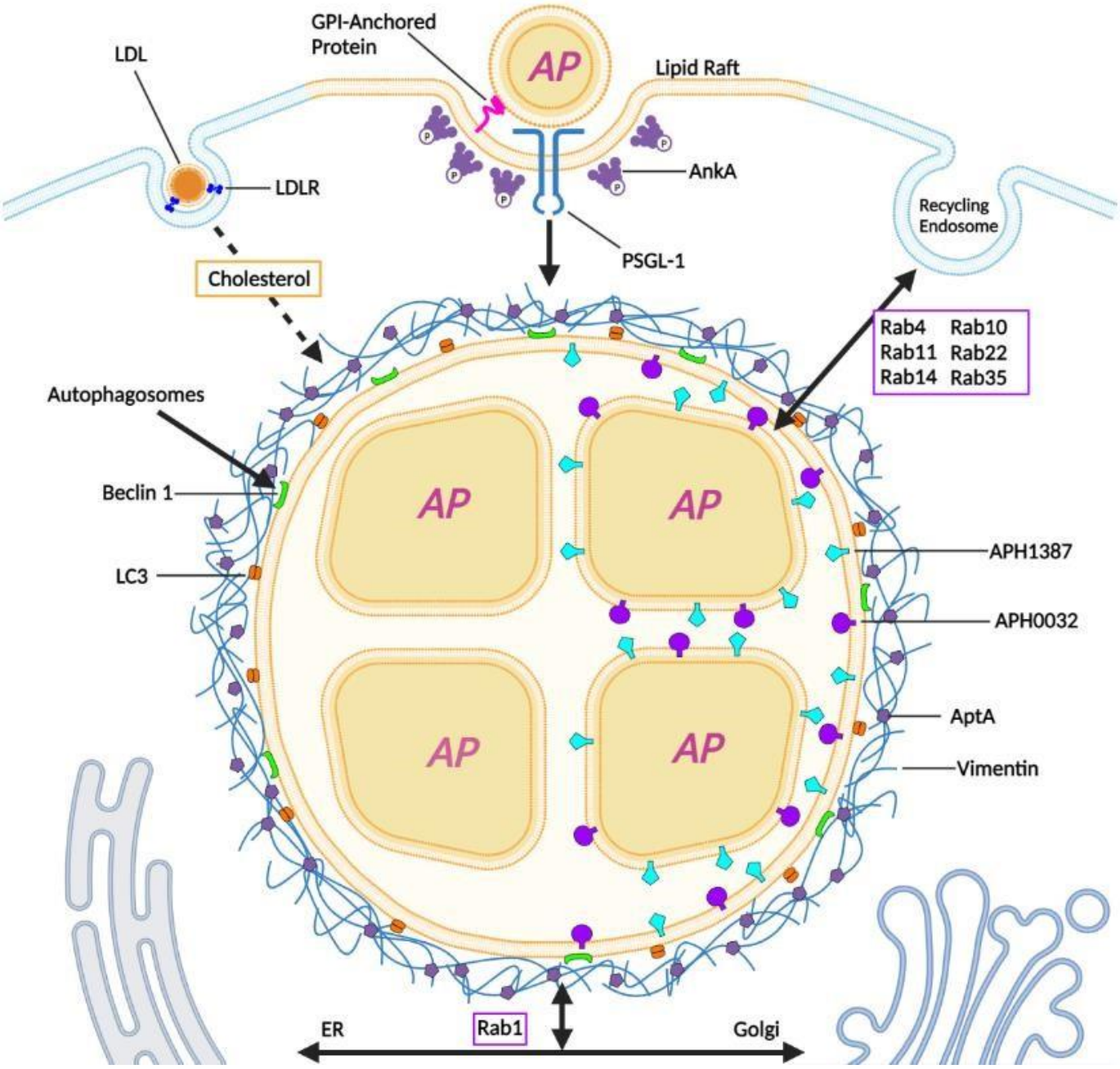

Figure 5. *A. phagocytophilum* morulae biogenesis. *A. phagocytophilum* binds to PSGL-1 (yellow). *A. phagocytophilum* hijacks host pathways during the formation of its vacuole. These pathways include endosome recycling endosomes by the manipulation of Rab proteins, autophagosome formation as evident by the colocalization ofLC3 and Beclin 1, and LDL uptake pathways to acquire host derived cholesterol. The filament protein vimentin interacts with *A.*

phagocytophilum toxin A (AptA) and decorates the morulae. APH_1387 and APH_0032 are also found in bacterial vacuole. During early infection, phosphorylated AnkA associates with the morulae. Reprinted from [67]. Created with BioRender.com.

2.2.2 *Anaplasma phagocytophilum* tick transmission

To infect a host, the cells of *A. phagocytophilum* must first colonize the tissues of their tick vector. This process appears to be reliant on factors produced by the tick. Colonization by *A. phagocytophilum* is mediated by the tick midgut antigen subolesin, as immunization against or silencing of this protein adversely effects bacterial numbers in the midgut [103, 104]. Many of the same salivary factors that play a role in *B. burgdorferi* transmission are also involved in the transmission of *A. phagocytophilum*. Differences arise in how these factors are utilized and modulated. For example, Salp16 is required for the establishment of *A. phagocytophilum* in the tick vector. Silencing of *Salp16* reduced the number of bacteria within ticks by 90% when compared to controls [105]. Additionally, the expression of the anticlotting tick proteins Salp9, Salp11 and thrombin inhibitors increases during *A. phagocytophilum* infection [106, 107]. Another anticlotting factor upregulated in the tick during *A. phagocytophilum* is a metalloprotease known as *Metis-1*, which is thought to stimulate fibrinolysis [108]. Also upregulated are the immune inhibitor proteins Sialostatin L and Sialostatin L2, as well as prolyl 4-hydroxylase subunits [109]. These proteins are known to facilitate *A. phagocytophilum* transmission by inhibiting the formation of the NLRC4 inflammasome, which is activated by components of the T4SS. By inhibiting the activation of the NLRC4 inflammasome, the production of caspase 1 is reduced, preventing the secretion of IL-1β and IL-18 by macrophages [110, 111]. Dissemination of the bacteria through the tick appears to be mediated by the tick salivary protein P11. This protein enables *A. phagocytophilum* to infect tick hemocytes, allowing

transport through the hemolymph to the salivary glands of the tick [112]. Silencing of the *P11* gene reduced *A. phagocytophilum* numbers in tick salivary glands compared to controls [113].

Mechanisms of infection within the tick vector of *A. phagocytophilum* are less understood when compared to the infection process in neutrophils. Tick cells have been used as model to understand vector-pathogen interactions. In a study looking at the transcriptional response of *I. scapularis* and *Ixodes 17icinus*, the vector of *A. phagocytophilum* in Europe, cell lines showed that ISE6 cells, *I. scapularis* embryonic cells, have a gene expression profile similar to infections in tick hemocytes when infected. On the other hand, *I. 17icinus* derived-cells, IRE/CTVM20, displayed a transcriptional response that was more similar to infection of tick midgut cells [114]. Similar to its development in neutrophils and HL-60 cells, *A. phagocytophilum* has a biphasic life cycle during infection of the cells [84]. α1,3-fucosylation has been shown to be involved in *A. phagocytophilum* colonization of ticks, potentially serving as a ligand. This was determined using the *Ixodes 17icinus* tick embryonic cell line IRE/CTVM19 and siRNA of α1,3fucosyltransferases in *I. scapularis* ticks [115].

3. Extracellular Vesicles and Vector-Borne Diseases

3.1 Extracellular vesicles classification and biogenesis

Cells, just like larger multi-cellular organisms, can communicate with nearby or distant neighbors. For cells, this is accomplished by several mechanisms. One way that cells communicate is through the production and secretion of extracellular vesicles. Extracellular vesicles are lipid bilayer packages containing transmembrane proteins and filled with cytosolic

proteins, DNA, and RNA. Cells can secrete different types of extracellular vesicles which are classified according to their sub-cellular origin, biogenesis, content and biochemistry [116, 117].

Microvesicles are formed and released through the budding of the cells' plasma membrane and range between 100–1,000 nm in diameter. A second type of extracellular vesicles known as exosomesare 150 nm or less in diameter and their biogenesis take place inside multivesicular endosomes termed "multivesicular bodies"[118]. Another group of vesicles known as apoptotic bodies are >1,000 nm in size, and are formed during the death of cells during apoptosis [119]. All extracellular vesicles display surface molecules that allow them to bind to their cellular recipient. Once received by the target cell, extracellular vesicles may induce the activation signaling pathways via receptor-ligand interaction [120]. They may also be internalized or even fuse with the target cell's membrane to deliver their content into its cytosol [119, 121].

Two pathways facilitate the formation of exosomes: the endosomal sorting complex required for transport (ESCRT)-dependent and the ESCRT-independent networks. The ESCRT-dependent pathway functions as a stepwise recruitment of molecular complexes (ESCRT 0, I, II, and III) to the endosomal membrane. These complexes function in the recognition of ubiquitylated proteins, the formation of the intraluminal vesicles, and their cleavage [120, 122]. The ESCRT-independent pathway functions through the interactions between sphingomyelin, acid sphingomyelinases (aSmases) and neutral sphingomyelinases (nSmases). These interactions hydrolyze sphingomyelin, producing ceramide at the membrane of the multivesicular body. This results in the budding of an exosome from the endosomal membrane into the multivesicular body [119, 123]. A recently discovered ESCRT-independent mechanism involves tetraspanins, specifically tetraspanin CD63, and can be initiated without ubiquitination, ESCRT, or ceramide [120]. The transport of the multivesicular body to the plasma membrane is accomplished

through the actions of the cytoskeleton, a molecular motor, and Rab GTPases, a Ras-like protein family associated with vesicular trafficking [124]. Once transported to its destination, it appears that soluble N-ethylmaleimide-sensitive factor activating protein receptors (SNAREs) act to release the contents of the multivesicular bodies [125].

Microvesicles are formed by the invagination of the plasma membrane stimulated with the accumulation Ca2+ [117]. The accumulation causes proteins known as flippases and floppases to rearrange the lipids in plasma membrane. The alterations of the cytoskeleton dynamics, which may work in conjunction with The ESCRT-III complex in the excision of microvesicles , though the exact method remains unknown [118, 126].

3.2 Extracellular vesicles in vector-borne diseases

Extracellular vesicles have been shown to induce a number of effects across the spectrum of vector-borne disease agents, allowing pathogens to influence the immune responses and genes of their host. Infection with the trypanosome *Trypanosoma cruzi*, the causative agent of Chagas disease, results in the pathogen producing vesicles that carry proteins associated with modulation of actin in its host's cells. This allows the trypanosome to successfully infect host cells and evade the host's complement system. The rickettsia *Orientia tsutsugamushi*, the causative agent of scrub typhus, produces extracellular vesicles associated with a 56-kDa protein implicated in host cell invasion [127]. Furthermore, arboviruses like the Dengue fever virus are known to effect host derived extracellular vesicles, with dengue infected *Aedes aegypti* mosquitoes produce vesicles containing effectors that enhance dengue virus infection [128, 129].

These vesicles can also act on the activation of host immune responses. Oxidized DNA molecules contained in *T. cruzi* extracellular vesicles are recognized by host macrophages,

leading to proinflammatory cytokine production [130, 131]. *Trypanosoma brucei*, the causative agent of sleeping sickness, also produces immunomodulatory extracellular vesicles responsible for aiding survival and invasion of host cells [130]. Furthermore, exosomes produced during *Orientia tsutsugamushi* infection had an altered microRNA profile and resulted in a proinflammatory effect when exposed to macrophages [132]. The use of these vesicles has been proposed as a method of vaccination against the bacterium [133].

Arthropod vectors also produce extracellular vesicles just like their associated pathogens. These vesicles appear to be used primarily for vector-host interactions, with studies showing that the proteins within these vesicles are associated with molecular functions such as proton transport, detoxification, ECM-receptor interaction, ribosomes, RNA transport, ABC transporters, and oxidative phosphorylation [12]. Studies utilizing the tick *Haemaphysalis longicornis* have shown that these vector-derived extracellular vesicles contain miRNAs that share similarities to host miRNAs, specifically let-7, miR-184, miR-375, miR-71 and bantam [134, 135]. These vesicles play a role in tick survival, as knockdown of miR-375 has been shown to result in a reduction of engorged body weight and a lack of embryo development for *H. longicornis*. Unfed *H. longicornis* nymphs show high expression of miR-375 suggesting it has a role in tick development and maturation. [136]. Studies have also found that these tick-derived salivary vesicles may enhance feeding via downregulation of CXCL12 and upregulation of IL-8, which acts to inhibit the wound healing process [11]. Tick borne viruses, have also been shown to alter the extracellular vesicles produced by ticks, facilitating their transmission from vector to host by packing viral RNA and proteins into secreted vesicles [137-139]. Thus, extracellular vesicles are an essential part of the vector-pathogen-host interaction, modulating each other to facilitate feeding, infection, and survival.

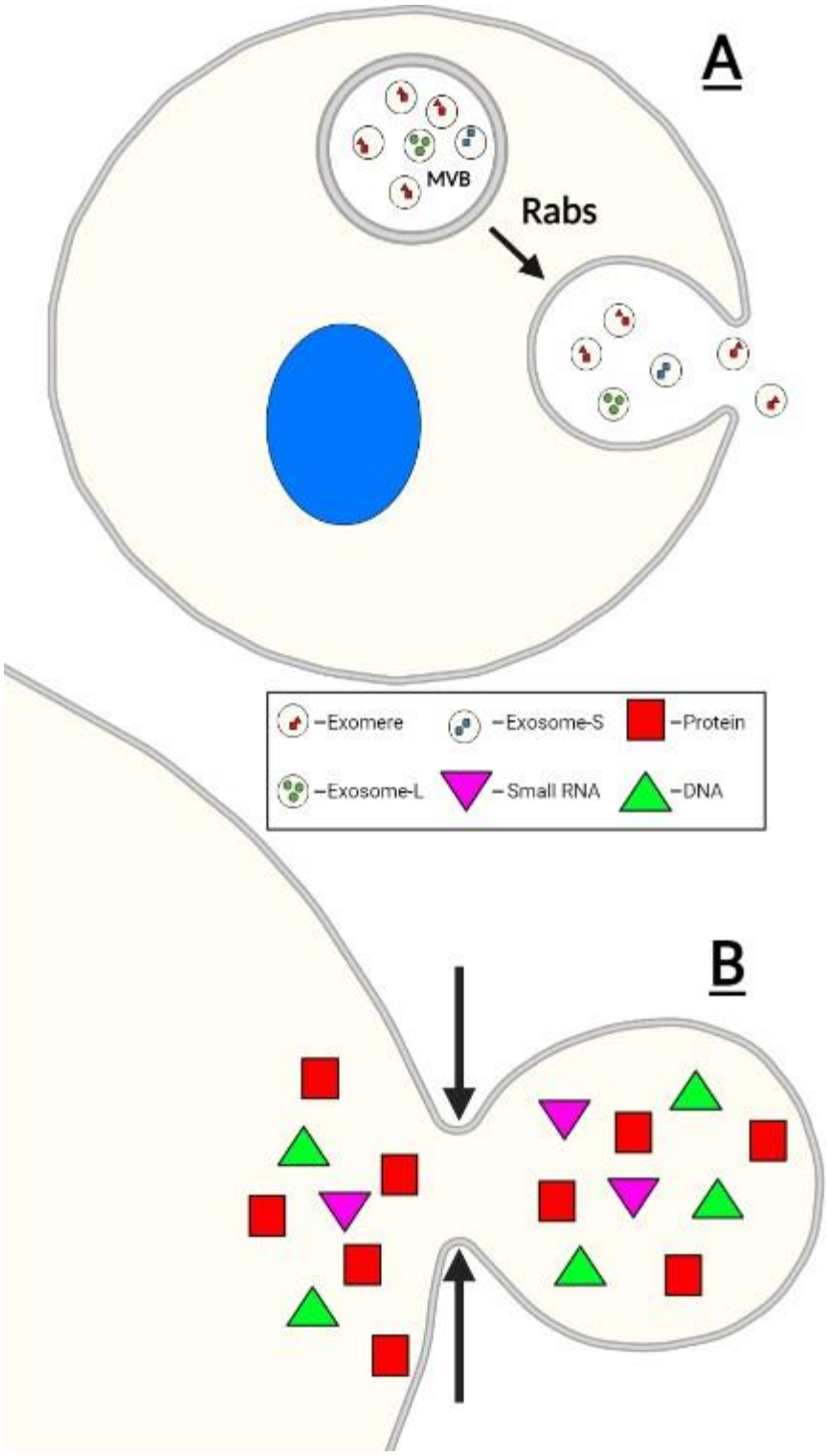

Figure 6. Extracellular vesicle biogenesis. (A) Exosomes are formed in multivesicular bodies.

Rab proteins mediate the transport of multivesicular bodies, and SNARE molecules facilitate the fusion of the vesicles with the plasma membrane, leading to their secretion from the cell. (B) Microvesicles are secreted through the invagination of the plasma membrane. Modified from

[118]. Created with BioRender.com.

4. Study Objectives

This project consists of three parts, aiming to further elucidate the effects of pathogen transmission on both the mammalian host utilizing a rodent model, as well as the tick vector. The goal of this project is to determine the markers of infection between infection with *A. phagocytophilum* and *B. burgdorferi*. The objectives of this project are:

1) Define the effect of tick-borne bacterial infection on the production of these vesicles by tick salivary glands and midguts.
2) Determine the changes in immune gene expression during transmission of *A. phagocytophilum*.
3) Compare differences in skin cytokine levels during *A. phagocytophilum* and *B. burgdorferi* transmission.

CHAPTER II

1. Introduction

The Black-Legged tick, *Ixodes scapularis*, has been spreading in recent years to more locations across the Northeastern and Midwestern United States. This tick is the primary vector of tickborne disease in the United States. As the vector of the human pathogens *Anaplasma phagocytophilum*, *Borrelia burgdorferi*, *Borrelia mayonii*, *Borrelia miyamotoi*, *Erlichia muris* sp. *eauclarensis*, *Babesia microti*, and Powassan virus, *I. scapularis* represents a major obstacle to public health. The pathogens *B. burgdorferi* and *A. phagocytophilum* represent the majority of locally acquired tick-borne illness in the US [140].

Several studies have looked at the immune response of the bite site during *B. burgdorferi* transmission. There, the upregulation of Th2 immune response is well documented, involving the secretion of cytokines and chemokines such as IL-5, IL-6, and IL-10 [141, 142]. During this time the associations between the tick salivary effector Salp15 and OspC plays a vital role in establishing infection, preventing CD4+ T cells activation and the phagocytosis *B. burgdorferi* spirochetes [43, 69-71].

For *A. phagocytophilum*, on the other hand, most studies have focused on systematic infections. Several studies have shown that IFN-γ produced during early phases of *A. phagocytophilum* infection are needed for control [143] and [144]. Infection of IFN-γ mutant mice leads to increased bacterial loads, but interestingly leads to reduced injury in organs [144]. While in the skin, *A. phagocytophilum* is associated with inflammation and inflammatory immune cells, neutrophils in particular [145]. Although several neutrophil effectors are dispensable for the

control of *A. phagocytophilum* [143]. Thus, whether these neutrophils facilitate or hinder infection is unknown.

Furthermore, both pathogens have been shown to affect the expression and secretion of salivary secretions by their vectors. *A. phagocytophilum* should be of note during these interactions, as unlike *B. burgdorferi*, it actively infects tick salivary cells to facilitate transmission [105]. Salivary effectors that are known to have an increased expression during both *A. phagocytophilum* and *B. burgdorferi* infection are Salp11 and Metis-1, an anticlotting factor and a putative stimulator of fibrinolysis, respectively [108, 109]. The anticoagulant prolyl 4hydroxylase is also upregulated by both pathogens [71]. Furthermore, just as with the *B. burgdorferi*-Salp15 interaction being essential for transmission, *A. phagocytophilum* transmission requires Salp16. This effector is necessary for the establishment of *A. phagocytophilum* within the tick [106, 146].

Herein, I describe the process undertaken to determine the changes that in gene expression and cytokine profiles during transmission of *A. phagocytophilum* and contrast it with the cytokine profiles during *B. burgdorferi* transmission. Additionally, the quantities of select cytokines in the skin and populations of extracellular vesicles produced by tick midguts and salivary glands will be quantified.

2. Methods

2.1 Cell culture.

HL60 Cells. HL60 cell cultures were maintained in RPMI media (Biological Industries, Cromwell, CT) supplemented with 10% Fetal Bovine Serum (Gibco, Waltham, MA), 1%

Glutamax (Gibco, Waltham, MA), and 1% amphotericin B (Gibco, Waltham, MA) and incubated at 37°C with 5% CO_2 [147, 148]. HL60 cells are a human leukemia cell line. These cells show a cellular morphology similar to that of neutrophils and are able to differentiate into granulocytelike cell types [149]. Cells were maintained until the cell density became optimal for passage or for infection with *A. phagocytophilum* (~1 to $5x10^5$ cells/ml). Cells were passaged as follows: 2 ml of cell culture were transferred into a 25 cm^2 tissue Falcon® culture flask (BD, Franklin Lakes, NJ) with 20 ml of freshly prepared culture media. This was repeated every 3 to 5 days, until cells reached passage 10 when they were discarded, and a new culture was recovered from liquid nitrogen (LN_2).

2.1.1 *Anaplasma phagocytophilum.*

For infection with *A. phagocytophilum*, 2 ml of uninfected HL60 cell cultures (at approximately $5x10^5$ cells/ml) were inoculated with 1 ml of *A. phagocytophilum* infected HL60 cells (at approximately $2x10^5$ cells/ml, with ~90% infection) for propagation. *A. phagocytophilum* was cultured in HL-60 cells for up to 5 days. Infections were monitored through post centrifugation of 1 ml of cell culture with a CytoSpin 4 (Thermo Scientific, Whaltham, MA) at 800xg for 5 minutes. Richard-Allan Scientific™ Three-Step Stain Kit (Thermo Scientific, Whaltham, MA) was use for staining. Staining was done by immersing the dried slide in fixative for 30 seconds, and then dipping the slide 15 times into step 1, then step 2 of the stain 15 times. The morulae within cells were observed utilizing light microscopy in a BX43F upright microscope (Olympus, Westborough, MA). Infections were passaged when the percentage of infection was greater than 80%, determined by counting a total of 100 HL60 cells and observing morulae. At this point 1ml of *A. phagocytophilum* infected HL60 cells were transferred to a new culture flask and 2 ml of uninfected HL60 cells were then added along with 17 ml of fresh culture media.

2.1.2 *Borrelia burgdorferi.*

B. burgdorferi MSK5 strain were cultured in BSKII medium with 50 ng/ml rifampicin (Fisher scientific, Waltham, MA) at 37℃ [150]. Passage of 1 ml of a mature culture into 9 ml of fresh culture medium was done when cell densities reached 180-200 cells per field and remained motile in a BX43F upright microscope with darkfield (Olympus, Westborough, MA) around 2-3 days post inoculation. The presence of all infectivity plasmids was monitored upon passage through boil prep to collect cellular DNA by collecting 1 ml of cell culture into a 1.5 ml Eppendorf tube, and placing the tube into a boiling water bath for 5 minutes. The samples were spun down for 2 minutes and the supernatant collected for PCR amplification of specific genes within each plasmid (Table 1). The presence of the circular plasmid (cp) 9, cp26 linear plasmid (lp) 25, lp28-1, lp28-2, lp28-3, lp28-4, lp35, lp54, and lp56 were confirmed using the primers listed in table 1. PCR was performed using the AccuStart II PCR SuperMix (QuantaBio, Beverly, MA), 1 ul of forward (F) and 1 ul reverse (R) primer at 10 M, and 2 ul of sample. The amplicons were amplified using the following conditions: denaturing for 1minute at 94℃ temperature, followed for 30 cycles of 20 seconds denaturalization, 30 seconds at an annealing temperature of 55℃ and an extension temperature of 68℃ for 2 minutes. The final amplification cycle was at 68℃ for 3 minutes. Without these plasmids, *B. burgdorferi* is unable to maintain an infection in a live host [32, 35, 151]

Table 1. Primers used for identification of *B. burgdorferi* infectivity plasmids in the MSK5 strain.

Name*	**Sequence**
BBC10 (cp9)	F-GAA CTA TTT ATA ATA AAA AGG AGA GC R-ATC TTC TTC AAG ATA TTT TAT TAT AC

BBB19	F-AAT AAT TCA GGG AAA GAT GGG
(cp26)	R-AGG TTT TTT TGG ACT TTC TGC C
BBD10	F-CAA ACT TAT CAA ATA GCT TAT C
(lp17)	R-ACT GCC ACC AAG TAA TTT AAC
BBE16	F-ATG GGT AAA ATA TTA TTT TTT GGG
(lp25)	R-AAG ATT GTA TTT TGG CAA AAA ATT TTC
BBF20	F-ATG AAC AAA AAA TTT TCT ATT TC
(lp28-1)	R-GTT GCT TTT GCA ATA TGA ATA GG
BBG02	F-TCC CTA GTT CTA GTA TCT ACT AGA CCG
(lp28-2)	R- TTT TTT TTG TAT GCC AAT TGT ATA ATG
BBH06	F-GAT GTT AGT AGA TTA AAT CAG
(lp28-3)	R-TAA TAA AGT TTG CTT AAT AGC
BBI16	F-GCA GGC CGG ATT TTA ATA TCG ATC
(lp28-4)	R-GCT CAT TAG ATA GCG TAT TTT TTA G
BBK19	F- AAG TTT ATG TTT ATT ATT GC
(lp36)	R-ATT GTT AGG TTT TTC TTT TCC
BBJ34	F-AAA TTC TAT GGA AGT GAT G
(lp38)	R-TTT CTA TTT ATT TTT AGG C
BBA16	F-GCA CAA AAA GGT GCT GAG
(lp54)	R-TTT TAA ACG GTT TTT AAG C
BBQ56	F-AAG ATT GAT GCA ACT GGT AAA G
(lp56)	R-CTG ACT GTA ACT GAT GTA TCC

*[152]

2.2 Animal infections.

Ten C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, ME USA) were used for pathogen acquisition due to their high susceptibility to infection from gram negative bacteria. These mice carry a mutation in their toll-like receptor 4 (Tlr4) gene, likely the cause of the enhanced susceptibility. When young, these mice show an increase in IgG titers and the occurrence of polyarthritis in *B. burgdorferi* infections, and a significant increase of monocytosis is observed 10 days post-infection with *A. phagocytophilum* when compared to immunocompetent mice [153]. Five mice were given 100 ul intraperitoneal injections of *A. phagocytophilum* in HL-60 cells at ~90% infection. Cells were spun down at 300 xg for 10 minutes, culture media was removed, and the remaining cells were suspended in 1x PBS. The number of bacteria was estimated using the following formula: TOTAL # OF BACTERIA = (TOTAL # OF CELLS) x (% INFECTION) x (5) x (19) [154]. The resulting inoculum contained $1x10^7$ bacteria/ml. Control mice received an injection of 100uL 1x PBS. Mice were injected with 27-gauge needles.

Cheek bleeds were performed on the mice on days 3, 5, and 7 collecting 50 to 100 ul of blood after anesthesia with 2%-1.25% isoflurane. 50 to 100 ul of blood was used for DNA extraction with the QIAGEN DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) and was completed by following the manufacturer's instructions . PCR analysis of the specific, multiplecopy *A. phagocytophilum p44* and the single *rpoB* genes was performed on the blood to confirm infection with *A. phagocytophilum*. Per reaction: Molecular grade H_2O 25.75 ul, GoTaq 0.25 ul (Promega, Madison, WI), 5x SYBR Green 10 ul, $MgCl_2$ 3 ul, dNTPs (NEB, Ipswich, MA), 1 ul, Primers Forward and Reverse 1 ul each, DNA template 8 ul. An annealing temperature of 56℃ (*p44*) and 53℃ (*rPOB*) was used for the reaction. Cycling conditions were as follow: 1 denaturing cycle for 3 minutes at 95°C, followed by 34 cycles of 1 minute denaturalization at

95°C, 1 minute at the annealing temperature (described above) and an extension of 72°C for 30 seconds. A final extension step of 5 minutes at 72°C was done. Predicted product sizes are displayed in Table 2.

2.2.1 *Borrelia burgdorferi* tick acquisition.

Larval *I. scapularis* ticks were infected with *B. burgdorferi* using an immersion technique [155]. Briefly, *I. scapularis* larvae were placed into a *B. burgdorferi* spirochete suspension. The larval ticks were cooled to 4°C and transferred as groups of 200 into 5 ml tubes and be left to warm to room temperature. 2 ml of *B. burgdorferi* culture at cell densities of 180-200 cells per field was then added to the tubes containing the larval ticks. The tubes were incubated at 32°C for 2 hours and vortexed every 10 minutes to maintain suspension of larvae in the medium. Following incubation at 32°C, the tubes containing the ticks were momentarily placed on ice and then centrifuged at 200 x g for 30 seconds. Supernatant was removed and the ticks were washed with PBS at 4°C. The larvae were left to dry for 24 hours. DNA was extracted from ~50 larvae with Chelex extraction to confirm infection [156]. The quality of the purification was assessed using an actin PCR: H_2O 10 ul, AccuStart "Tough Start" (Quantabio, Beverly, MA) PCR 10 ul, Primers F/R 1 ul each, DNA template 5ul. Once the extracted DNA was confirmed, a PCR utilizing the *B. burgdorferi* gene *recA* was then performed on the larvae. H_2O 10 ul, AccuStart "Tough Start" PCR 10 ul, Primers F/R 1 ul each, DNA template 5ul. PCR amplification conditions were as follows: An annealing temperature gradient of 55°C was used for the reaction. Cycling conditions were: 1 denaturing cycle for 1 minute at 94°C, followed by 30 cycles of 20second denaturalization at 94°C, 30-seconds at the annealing temperature (described above) and an extension of 68°C for 2 minutes. A final extension step of 3 minutes at 68°C was done. Predicted sizes of PCR products are displayed in Table 2.

2.2.2 *Anaplasma phagocytophilum* tick acquisition

For *A. phagocytophilum* infections, 200 larval *I. scapularis* ticks were placed on mice after confirming infections. Mice were separated into individual mesh bottom cages placed above a water trap to collect the fed ticks. Mice were anesthetized for 30 minutes with 2%-1.25% isoflurane to allow the larvae to attach. Engorged ticks were collected from the water baths after 3, 4 and 5 days of feeding. The mice were euthanized with CO_2, followed by exsanguination and cervical fracture. The collected ticks were washed in 2% bleach and autoclaved water, placed into groups of 25, and allowed to molt into nymphs. DNA was extracted from 3 molted nymphs using Chelex extraction. PCR of the *B. burgdorferi recA* and *A. phagocytophilum rpoB, msp5* and *p44* genes were performed as described above.

2.3 Pathogen Transmission.

The infected nymphs were used to infest C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME), which have a proper functioning immune system with no known mutations in their immune related genes. Twenty-five *I. scapularis* nymphs were placed on each mouse. Mice were anesthetized as previously described for the larvae. The ticks were allowed to feed for 3 days, the minimum time required for successful transmission of *A. phagocytophilum* and *B. burgdorferi* from the tick to the host [157, 158]. After this time, the mice were sacrificed with CO_2, followed by cervical fracture. Three (3) mm skin biopsies were taken of the bite sites utilizing Integra Miltex disposable biopsy punches (Integra Miltex, Plainsboro, NJ) for cytokine detection and RNAseq. Samples containing only single infections of either pathogen (i.e., only *A. phagocytophilum* or *B. burgdorferi*) were used for cytokine detection, and only *A.*

phagocytophilum infected samples were sent out for RNAseq. RNAseq samples were put in RNAlater (ThermoFisher Invitrogen, Waltham, MA) and stored at -80 °C, whereas skin samples used for cytokine measurements were flash frozen with dry ice. The partially engorged nymphs were collected for excision of their midguts and salivary glands.

Table 2. Depiction of the primers used and their sequences.

Name	Sequence	Predicted Size	Reference
Mouse Actin	F- ACG CAG AGG GAA ATC GTC GAC R-ACG CGG CAG GAA GAG GAT GCG GCA GTG		[154]
Ixodes Actin	F-GGT CAT CAC AAT CGG CAA R-ATG CAG TTG TAC GTG GTC TC	108	[13]
RecA *Borrelia burgdorferi*	F-GTG GAT CTA TTG TAT TAG ATG AGG CT R-GCC AAA GTT CTG CAA CAT TAA CAC CT	222	[154]
P44-18ES	F-GGT GTG TGA GAC AAA GCG G R-CTG GAC GTA GGC CAG TTC T	216	[159]
rPOB ApH.1024	F-CTT TAT CCT GCT TTA GAA CAA CAT C R-GGT CCG TAT GGT CTG GTT ACT	286	[160]
MSP5	F-TGA CAC TGT GGT TGA ACA AGC R-GAA GAA AAG CCG AAC ATA AGC	126	[160]
MSP4	F-CGT CTG ATG TTA GCG GTG R-TTA GCG AAC TTG AAT GAG G	205	[160]

2.4 Cytokine detection at the bite site.

Skin biopsies were processed by homogenization on ice with 500 ul Bioplex Cell Lysis Buffer (Bio-Rad, Hercules, CA) with 0.05% Bovine Serum Albumin. ELISA MAX kits (BioLegend, San Diego, CA) were be used to identify cytokines in skin biopsies collected from bite sites. Manufacturer protocols were followed with a few modifications. ELISA MAX Uncoated Plates (Biolegend, San Diego, CA) were incubated with the detection antibody overnight at 4°C. The next day, wells were washed with PBS with 0.5% TWEEN 3 times and 100 ul of samples were incubated in duplicate overnight at 4°C with shaking. The following day, detection antibodies, Avidin Horseradish Peroxidase (HRP) solution, and 3,3',5,5'-Tetramethylbenzidine substrate (TMB) were added after 3-4 washes. Plates were incubated with TMB substrate for 20 minutes. The plates were read in a TECAN I-Control imager using the Magellan software program (Tecan, Männedorf, Switzerland) at 450 and 570 nm. Statistical analysis was conducted with an ANOVA, using the Graphpad Prism program (Graphpad, San Diego, CA).

Table 3. Cytokines examined in experiments and their recognized functions.

Cytokine/Chemokine	**Function**
IL-2	Proliferation and differentiation of T cells, including regulatory T cell development and survival; proliferation and activation of Natural Killer (NK) cells [161].
IL-5	Eosinophil activation, B-cell differentiation, increased eosinophil and B-cell generation [162].

GM-CSF	Maturation of granulocytes and monocytes, activation of macrophages [163].

2.5 Extracellular vesicle free media

L15C-300 medium containing 5% FBS (Fetal Bovine Serum; ThermoFisher Invitrogen, Waltham, MA), 5% TPB (Tryptose Phosphate Broth; Sigma-Aldrich, Saint Louis, MO), 1% LPC (Lipoprotein Cholesterol; MP Biomedicals, Irvine, CA), 5% $NaHCO_3$ (Sigma-aldrich, St. Louis, MO), 2.5% HEPES buffer (Sulfonic Acid; Sigma-aldrich, St. Louis, MO), and 81.5% diluted L15C is centrifugated at 100,000 xg for 18 hrs. Supernatant is collected and spun again at 100,000 xg for 18 hrs, and the supernatant collected again. This is done to clear the media of any extracellular vesicles already present in the media upon preparation. After preparation 1x amphotericin B, 1x Penicillin (MP Biomedicals, Irvine, CA), 1x Gentamicin (Gibco, Waltham, MA), and 1x Cefotaxime (Millipore Sigma, Burlington, MA), are added to prevent microbial contamination [164, 165].

2.6 Salivary gland and Midgut cultures

Salivary gland cultures were maintained as in previous studies [13, 166]. Briefly, nymphal *I. scapularis* ticks were immobilized utilizing carpet tape adhered to a microscope slide and dissected in PBS, using 4 mm Vannas small scissors (BVI Medical, Waltham, MA) intended for ocular surgery. The salivary glands and midguts were removed and placed in 500 ul extracellular vesicle free media. Organs were placed groups together in a 42 well plate and incubated at 34 °C for 24 hrs to allow vesicle secretion.

2.7 Extracellular vesicles purification

The samples containing the extracellular vesicles were collected and centrifuged for 10 minutes at 300 x g at 4 °C. The supernatant was centrifuged again for 10 minutes at 2,000 x g at 4 °C. The supernatant was then centrifuged again for 30 minutes at 10,000 x g at 4 °C. Vesicles were collected in Vivaspin 500 (Sartorius, Stonehouse, UK). Collected vesicles were stored in 1x PBS at -80°C for later analysis.

2.8 Extracellular vesicles NTA measurement

Measurement of extracellular vesicle concentrations from infected and uninfected ticks was done with the Nanosight LM10 (Malvern, United Kingdom). This device makes use of light scattering and Brownian motion to obtain the size and concentration of suspended particles. A laser beam is passed through the sample, scattering the light, allowing the particles to be easily visualized with 20x magnification. A camera that operates at 30 frames per second (fps), captures a video of the field of view, and installed software tracks the particles and uses the Stokes-Einstein equation to calculate their hydrodynamic diameters. Samples were diluted at a 1:20 and read three times for accurate at a capture of screen gain: 4; camera level: 7, and measurement at screen gain: 17; detection threshold: 3. Statistical analysis was conducted with a two-way ANOVA.

2.9 Transcriptome at the bite site

3 mm skin punches were taken from the C57BL/6J mice were put inside 0.2 ml tubes containing 50 ul RNALater reagent (ThermoFisher Invitrogen, Waltham, MA) and stored at -80°C. Samples were collected from a group of non-infested mice, to serve as a baseline and statistical control. Skin collections from the bite sites of *Anaplasma phagocytophilum* infected ticks, and uninfected ticks were submitted to GeneWiz (South Plainfield, NJ) for RNA isolation. Samples were mailed

overnight via FedEx in an insulated box filed with dry ice. Total RNA extraction was performed using the Qiagen RNeasy Plus Universal mini kit (Qiagen, Hilden, Germany), RNA quantification was accomplished using the Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA) and the integrity of the RNA was confirmed through utilization of the Agilent TapeStation 4200 (Agilent Technologies, Palo Alto, CA). RNA sequencing libraries were prepared using the NEBNext Ultra II RNA Library Prep Kit (NEB, Ipswich, MA), and validated with the Agilent TapeStation (Agilent Technologies, Palo Alto, CA). RNA samples were sequenced with a 2 x 150bp Paired End (PE) configuration. Image analysis and base calling were done through use of the HiSeq Control Software (HCS).

Sequences were trimmed for the removal of adapter sequences and poor-quality nucleotides. Trimmed reads were mapped to the reference genome available on ENSEMBL using the STAR aligner v.2.5.2b.

2.10 Ethics statement

All experiments were carried out under the protocols approved by the Institutional Biosafety (IBC2019-078) and Animal Care and Use committees (IACUC2019-0194) at Texas A&M University.

3. Results

3.1 *Borrelia burgdorferi* Plasmid Screening

The presence of all infectivity plasmids in the *B. burgdorferi* cultures was confirmed by PCR amplification of selected genes within each plasmid. Cultures lost their plasmid at variable rates with some cultures quickly losing plasmids (~3 passages), and others maintaining plasmids for a long period of time (10-12 passages). A gel from representative cultures is shown in Figure 1. In this case, of the three cultures maintained for producing infection two returned positive results, with cultures 1 and 2 returning positive visualization of all 12 infectivity plasmids. Culture one

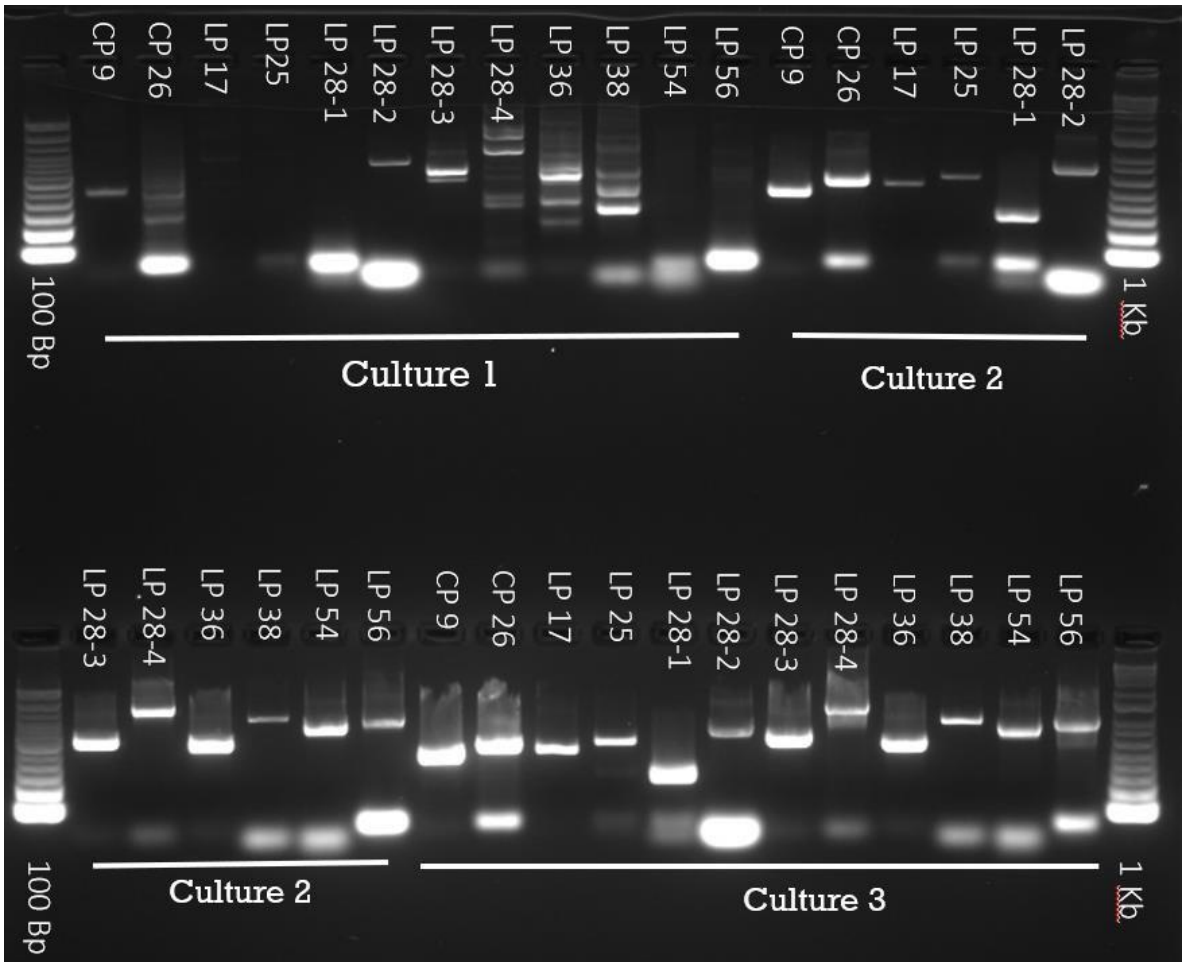

Figure 7. Image of the nucleotide gel electrophoresis of the *B. burgdorferi* infectivity plasmids. Three cultures are displayed. Cultures 2 and 3 display a positive result for all 12 infectivity plasmids. Culture 1 shows negative results in LP17, LP25, LP28-1, LP54 and LP56.

shows loss of 5 infectivity plasmids, so this culture was discarded and not used. This process was repeated multiple times before using any culture during experimentation.

3.2 Confirmation of Tick Infection

An immersion technique was used to infect larvae with *B. burgdorferi*. A preliminary experiment that involved bathing larval ticks in 1.5 ml of media at 37°C for 1, 1.5, 2, 2.5, and 3 hours and vortexing every 10 minutes determined that submersion of larval ticks in *B. burgdorferi* culture media ready for passage (~200 cells per field) for 2.5 hours were the optimal conditions for infection of the larvae. Following this experiment, all subsequent *B. burgdorferi* infections were performed as described in the materials and methods. We determined that ~ 20% of larvae became infected and retained the bacteria after molting (Figure 2). Of the five groups of larval ticks that were infected and fed to repletion, only one group was able to maintain infection with *B. burgdorferi* post-molt. These ticks were used for infesting mice for collection of bite site skin biopsies. The figure shown is representative of observations and are part of a process repeated four times.

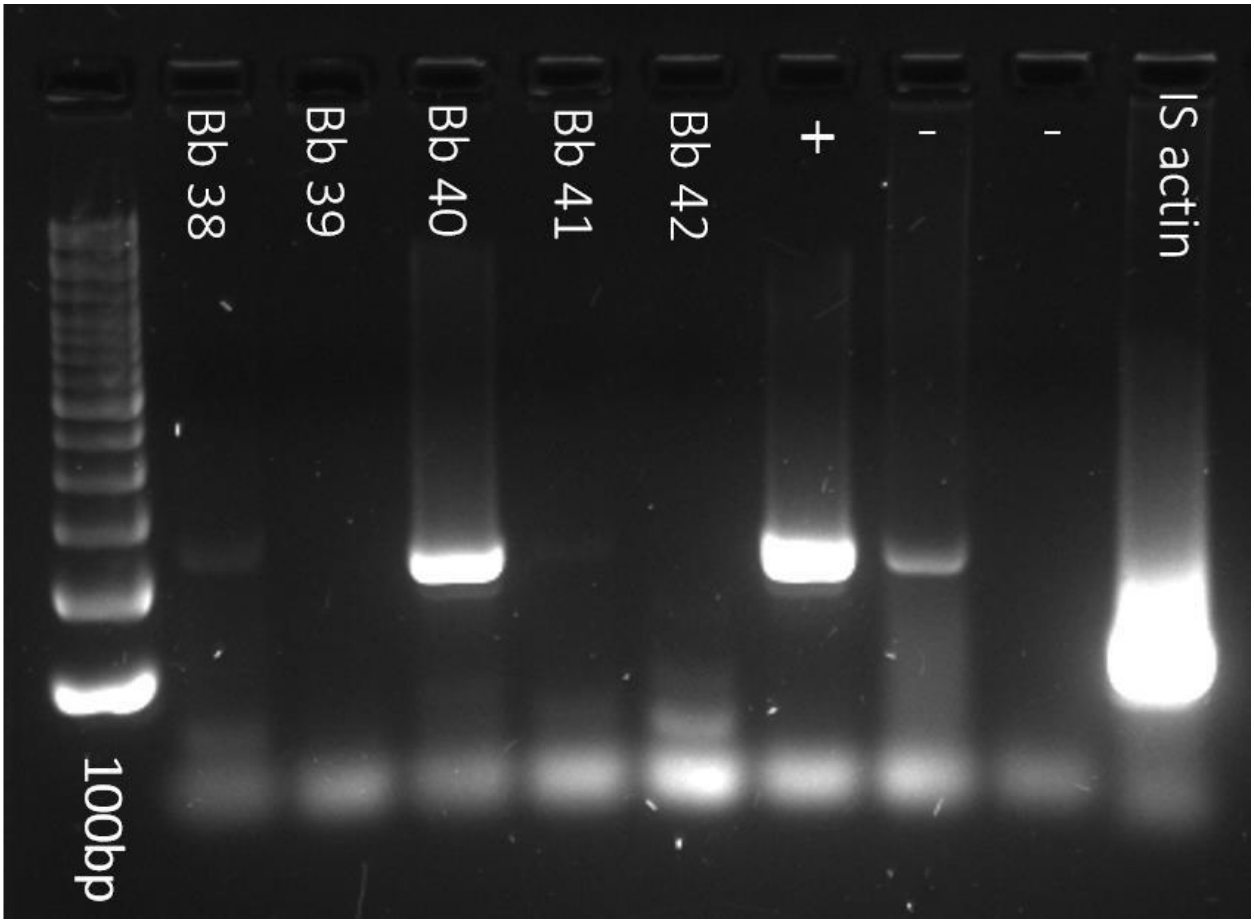

Figure 8. Image of the nucleotide gel electrophoresis of the *B. burgdorferi* nymphs following feeding and molting as larvae. Group Bb 40 maintained infection post-molt, as confirmed through a positive amplification of the *B. burgdorferi RecA* gene.

Nymphs that were fed on *A. phagocytophilum* infected mice as larvae were examined post-molt to determine if infection with *A. phagocytophilum* was successfully acquired by the ticks. PCR analysis of *A. phagocytophilum Msp5* was performed as described in the methods. Analysis showed infection in all groups of ticks tested (Figure 3). These ticks were used for infesting mice for collection of bite site skin biopsies. The figure shown is representative of observations and are part of a process repeated how many times.

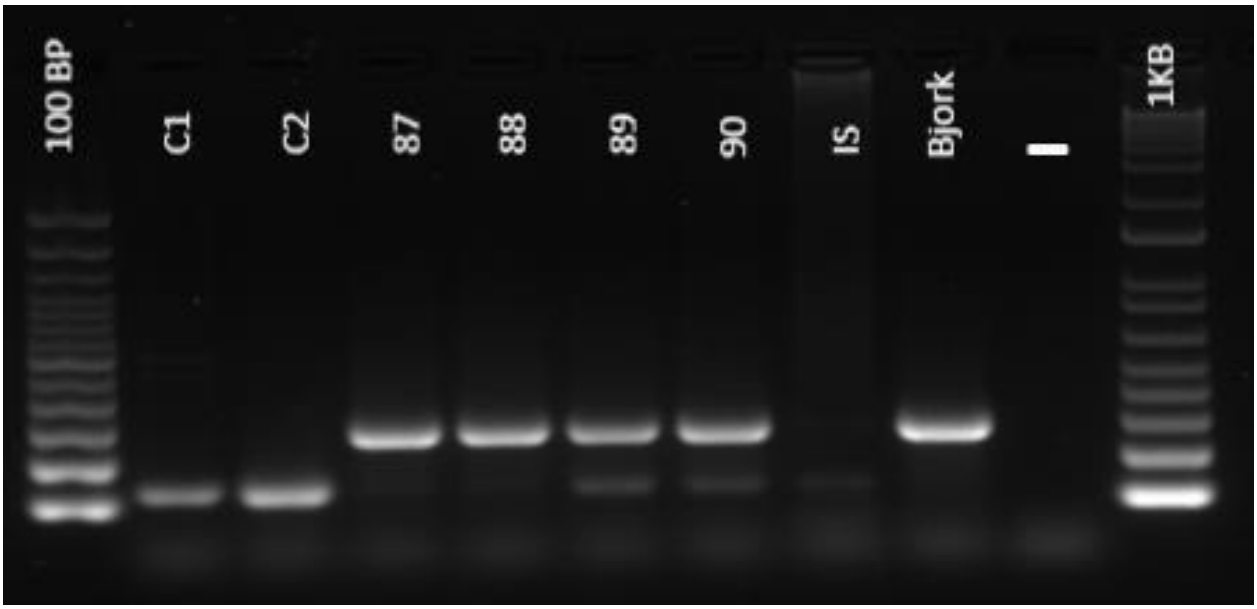

Figure 9. Image of the nucleotide gel electrophoresis of the *A. phagocytophilum* nymphs following feeding and molting as larvae. Tick groups 87, 88, 89, and 90 show positive infection, while control ticks C1 and C2 are negative for infection.

3.3 Changes in gene expression during tick feeding and pathogen transmission

Biopsies were collected from mice without ticks to determine the baseline expression, and these results were compared to biopsies taken from mice fed upon by uninfected ticks and *A. phagocytophilum* infected ticks. A total of 1,213 genes were differentially expressed during feeding by uninfected ticks. Of these genes, 797 were upregulated and 416 were downregulated. Of the differentially expressed genes induced by tick feeding, 40 were cytokines or chemokines (Table 4). Broadly, differentially regulated genes are related to the immune response, with genes associated with neutrophil chemotaxis, chemotaxis, inflammation, and immune system processes being the most significant as based upon gene ontology terms tested using the Fisher exact test

(GeneSCF v1.1-p2) (Figure 10). Upregulated genes include: *Cxcl5* (C-X-C motif chemokine 5), *Cxcl2, Il6* (Interleukin 6), *Cxcl3, Il1b, Il21r, Cxcr2, Cxcl1, Ccl7* (chemokine (C-C motif) ligand 7), *Ccl2, Ccl3, Ccl4, Ccr5, Ifng* (interferon gamma), *Ccr1, Ccr7, Csf3r* (colony-stimulating factor 3 receptor), *Ccl12, Ccl17, Ccl5, Cxcl10, Il4ra, Ccr2, Cxcl9, Il27, Ccl9, Ccl6, Ccl22, Cxcr4, Il2rg, Ccl8, Il18rap, Csf2rb, Il10, Il10ra, Il33,* and *Il12rb1*. Likewise, toll-like receptor genes *Tlr13, Tlr1,* and *Tlr6* were upregulated. Three immune genes were downregulated: *Il31ra, Il12a,* and *Il22ra*.

Table 4. Expression of select immune genes in the skin when an uninfected tick feeds compared to the baseline skin without a tick.

Gene	Description	log2FoldChange	P-value
Cxcl5	C-X-C motif chemokine 5	6.91	0
Cxcl2	C-X-C motif chemokine 2	4.53	0
Il6	Interleukin 6	4.34	0
Cxcl3	C-X-C motif chemokine 3	4.05	0
Il1b	Interleukin 1 beta	3.8	0
Il21r	Interleukin 21 receptor	3.8	0
Cxcr2	C-X-C motif chemokine receptor type 5	3.78	0

Cxcl1	C-X-C motif chemokine 1	3.77	0
Ccl7	chemokine (C-C motif) ligand 7	3.17	0
Ccl2	chemokine (C-C motif) ligand 2	3.03	0
Ccl3	chemokine (C-C motif) ligand 3	2.88	0
Ccl4	chemokine (C-C motif) ligand 4	2.73	0
Ccr5	chemokine (C-C motif) receptor 5	2.54	0
Ifng	IFN-gamma	2.47	0
Ccr1	chemokine (C-C motif) receptor 1	2.33	0
Ccr7	chemokine (C-C motif) receptor 7	2.27	0
Csf3r	colony stimulating factor 3 receptor (granulocyte)	2.24	0
Ccl12	chemokine (C-C motif) ligand 12	2.19	0

Ccl17	chemokine (C-C motif) ligand 12	2.08	0

Ccl5	chemokine (C-C motif) ligand 5	2.02	0
Cxcl10	C-X-C motif chemokine 10	1.83	0
Il4ra	Interleukin 4 receptor alpha	1.66	0
Ccr2	chemokine (C-C motif) receptor 2	1.62	0
Cxcl9	C-X-C motif chemokine 9	1.54	0
Il27	Interleukin 27	1.53	0
Ccl9	chemokine (C-C motif) ligand 9	1.52	0.01
Ccl6	chemokine (C-C motif) ligand 6	1.43	0
Ccl22	chemokine (C-C motif) ligand 22	1.41	0
Cxcr4	C-X-C motif chemokine receptor type 4	1.38	0

Il2rg	interleukin 2 receptor, gamma chain	1.38	0

Ccl8	chemokine (C-C motif) ligand 8	1.33	0
Il18rap	interleukin 18 receptor accessory protein	1.29	0
Csf2rb	colony stimulating factor 2 receptor subunit beta	1.22	0
Il10	Interleukin 10	1.21	0
Il10ra	Interleukin 10 receptor alpha	1.19	0
Il33	Interleukin 33	1.18	0
Il12rb1	Interleukin 12 receptor, beta 1	1.06	0
Il31ra	Interleukin 31 receptor A	-1.1	0
Il12a	interleukin 12a	-1.22	0
Il22ra2	interleukin 22 receptor, alpha 2	-1.52	0

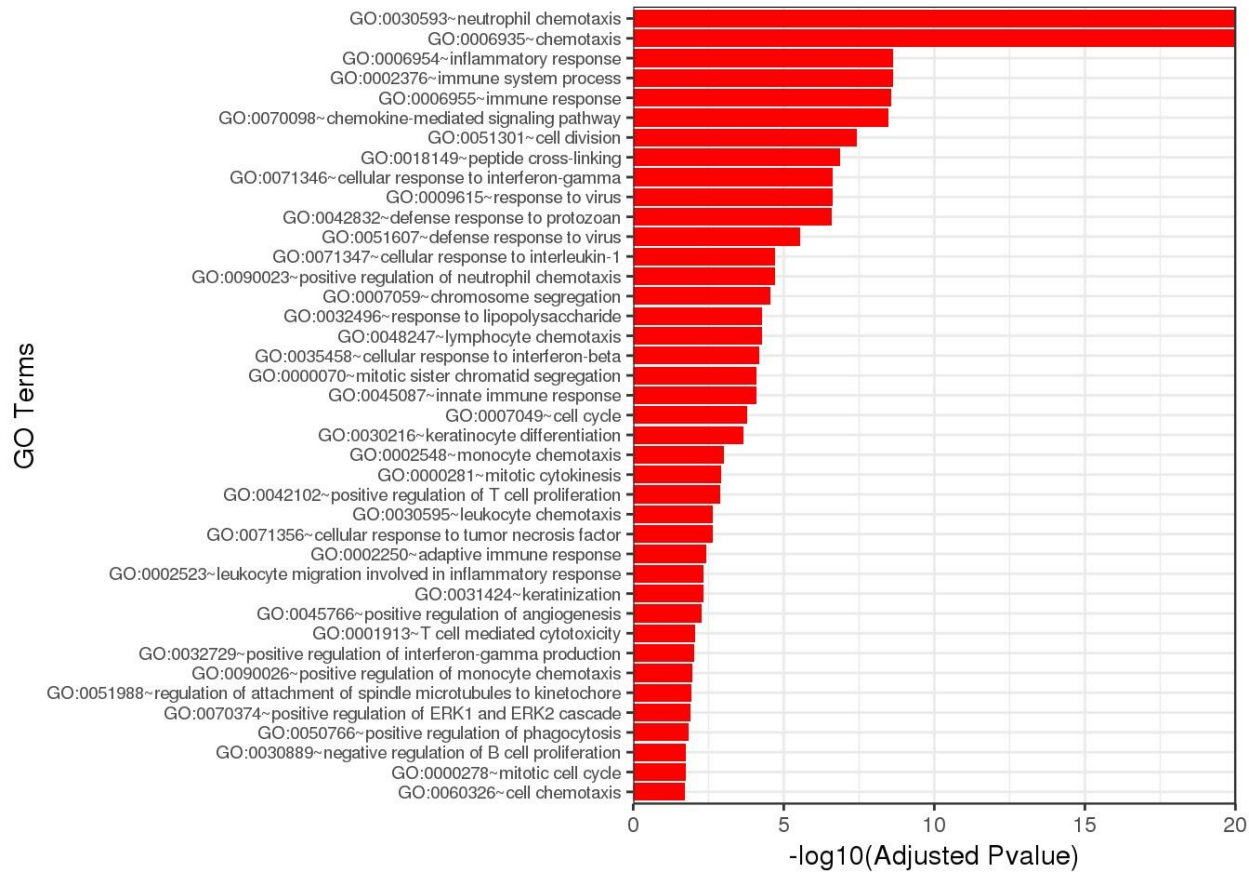

Figure 10. Gene ontology Enrichment of Baseline v. Control samples. Showing terms that are significantly enriched with an adjusted P-value of less than 0.05.

Comparisons to ticks infected with *A. phagoctophilum* were also performed. A total of 2,559 genes were differentially expressed during feeding with the infected tick compared to the baseline. Of these genes, 1,417 were upregulated and 1,142 were downregulated. Of the upregulated genes, a total of 43 were cytokines or chemokines (Table 5). In general, differentially regulated genes are immune response related genes, with genes associated with immune responses to viruses and protozoans, interferon gamma, and inflammation being the most significant as based upon gene ontology terms tested using the Fisher exact test (GeneSCF v1.1-p2) (Figure 11). Cytokines and chemokines upregulated from the baseline include: *Csf3*,

Ccl9 (Chemokine (C-C motif) ligand 9), *Ccl6*, *Ccl1*, *Ccl8*, *Cxcl5*, *Cxcr2*, *Cxcr4*, *Cxcl14*, *Il18rap*, *Il1f6*, *Il4ra*, *Il33*, *Il2rb*, *Il1f9*, *Il17rd*, *Il1r2*, *Il22ra2*, and *Il12a*. Genes for toll-like receptors were also differentially regulated, with 8 upregulated genes, and *Tlr5* being downregulated.

Table 5. Expression of select upregulated immune genes in the skin when an *A. phagocytophilum* infected tick feeds compared to the baseline skin without a tick.

Gene	Description	log2FoldChange	P-value
Cxcl2	Chemokine (C-C motif) ligand 2	8.04	0
Cxcl5	Chemokine (C-C motif) ligand 5	7.8	0
Il24	interleukin 24	7.51	0
Ccl4	Chemokine (C-C motif) ligand 4	6.84	0
Csf3	colony stimulating factor 3 (granulocyte)	6.42	0
Cxcl3	Chemokine (C-C motif) ligand 3	6.38	0
Ifng	Interferon-gamma	6.19	0
Cxcl10	Chemokine (C-C motif) ligand 10	6.07	0
Il6	interleukin 6	5.85	0
Il1b	interleukin 1 beta	5.77	0

Ccl5	Chemokine (C-C motif) ligand 5	5.66	0
Ccl3	Chemokine (C-C motif) ligand 3	5.58	0
Cxcl11	Chemokine (C-C motif) ligand 11	5.55	0
Cxcl1	Chemokine (C-C motif) ligand 1	5.23	0
Ccl2	Chemokine (C-C motif) ligand 2	5.14	0
Il21r	interleukin 21 receptor	5.09	0
Ccl12	Chemokine (C-C motif) ligand 12	5	0
Il27	interleukin 27	4.92	0
Ccl7	Chemokine (C-C motif) ligand 7	4.73	0
Cxcr2	C-X-C motif chemokine receptor 2	4.51	0
Cxcl9	Chemokine (C-C motif) ligand 9	4.42	0

Csf3r	colony stimulating factor 3 receptor	3.44	0
	(granulocyte)		
Il12rb1	interleukin 12 receptor, beta 1	3.42	0
Il10	interleukin 10	2.65	0
Il12b	interleukin 12b	2.64	0
Il2rg	interleukin 2 receptor, gamma chain	2.63	0
Il10ra	interleukin 10 receptor, alpha	2.5	0
Il18rap	interleukin 18 receptor accessory protein	2.28	0
Il19	interleukin 19	2.27	0
Il1f6	interleukin 36A	2.17	0
Il4ra	interleukin 4 receptor, alpha	1.96	0
Ccl9	Chemokine (C-C motif) ligand 9	1.71	0

Ccl6	Chemokine (C-C motif) ligand 6	1.61	0
Il33	interleukin 33	1.61	0
Csf2rb	colony stimulating factor 2 receptor, beta, low-affinity (granulocytemacrophage)	1.5	0
Cxcr4	C-X-C motif chemokine receptor 4	1.39	0
Il2rb	interleukin 2 receptor, beta chain	1.36	0
Il15	interleukin 15	1.35	0
Csf2ra	colony stimulating factor 2 receptor, alpha, low-affinity (granulocytemacrophage)	1.34	0
Ccl1	Chemokine (C-C motif) ligand 1	1.06	0
Ccl8	Chemokine (C-C motif) ligand 8	1.05	0

Cxcl14	Chemokine (C-C motif) ligand 14	1.02	0

Cytokines and chemokines downregulated from the baseline include: *Il17rd*, *Il1r2*, *Il22ra2*, *Ccl24*, and *Il12a* (Table 6).

Other immune related genes detected include the upregulated toll-like receptors *Tlr13*, *Tlr1*, *Tlr9*, *Tlr12*, *Tlr6*, *Tlr7*, *Tlr3*, and *Tlr2*. One toll-like receptor showed downregulation, *Tlr5*. This toll-like receptor is known to detect bacterial flagellin.

Table 6. Expression of downregulated immune genes in the skin when an *A. phagoctophilum* infected tick feeds compared to the baseline skin without a tick.

Gene	Description	log2FoldChange	P-value
Il17rd	interleukin 17 receptor D	-1.02	0
Il1r2	interleukin 1 receptor, type II	-1.05	0
Il22ra2	interleukin 22 receptor, alpha 2	-1.53	0
Ccl24	Chemokine (C-C motif) ligand 24	-1.74	0
Il12a	interleukin 12a	-2.47	0

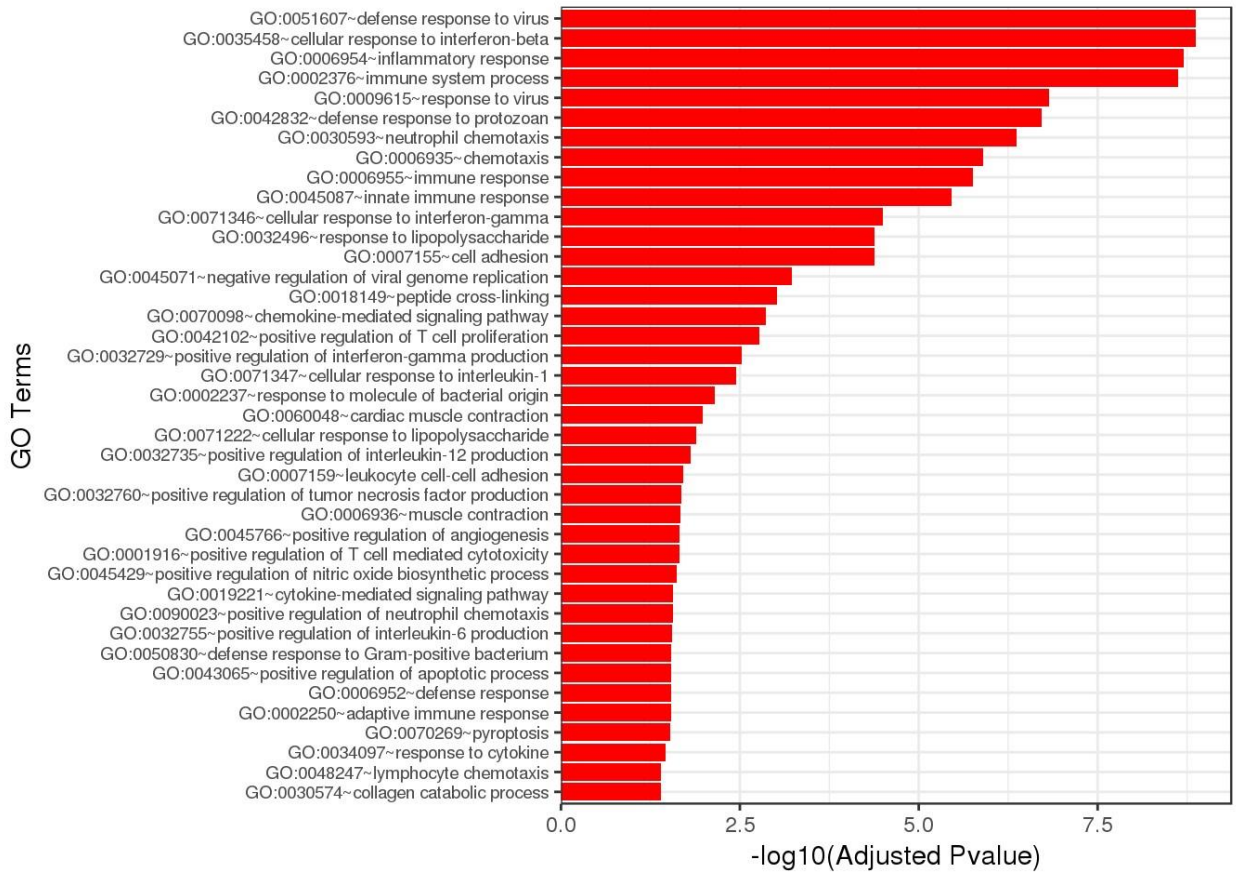

Figure 11. Gene ontology Enrichment of Baseline v. *Anaplasma* samples. Showing terms that are significantly enriched with an adjusted P-value of less than 0.05.

3.4 Effects of *A. phagocytophilum* transmission at the Bite Site

To determine the effect of pathogen transmission at the skin level, we used RNAseq of skin punch biopsies from 28 mice fed upon by uninfected ticks and ticks infected with *A. phagoctophilum*, which were collected earlier in this experiment. It was observed that of the 622 genes were differentially expressed during *A. phagocytophilum* infection, with 476 upregulated genes and 146 down regulated genes. As with the comparison with the baseline, differentially regulated genes are related to the immune response, with genes associated with immune responses to viruses and protozoans, interferon gamma, and inflammation being the most

significant as based upon gene ontology terms tested using the Fisher exact test (GeneSCF v1.1p2) (Figure 12). Downregulated genes primarily consisted of collagens and other extracellular matrix related genes (Table 8). Several chemokines were upregulated during the transmission of *A. phagocytophilum*, including *Ccl4*, *Ccl5*, *Ccl3*, *Ccl2*, and *Ccl7*. These chemokines are involved in the chemotaxis of immune cells to sites of acute inflammation. The only chemokine showing downregulation was *Ccl24*, a chemokine also related to chemotaxis in several immune cell types, including neutrophils, lymphocytes, monocytes, and eosinophils (Figure 13).

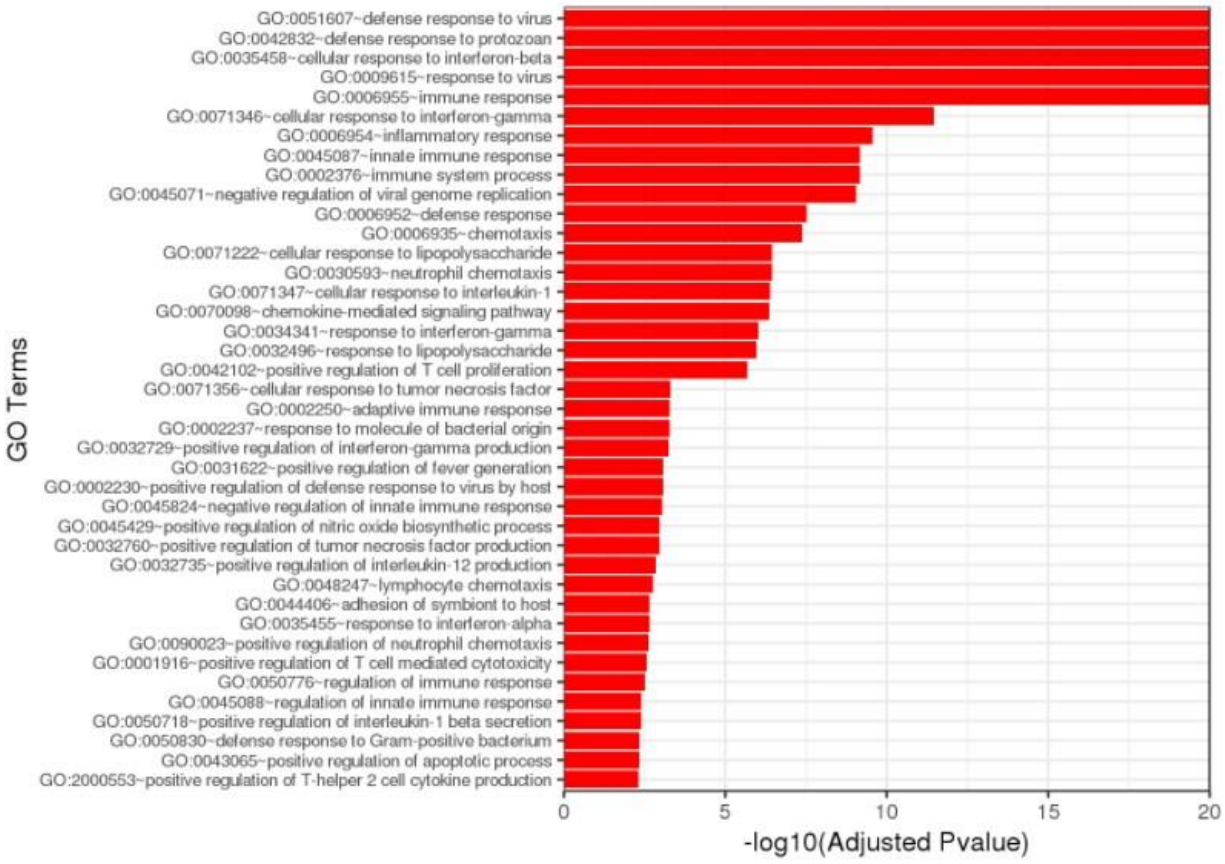

Figure 12. Gene ontology Enrichment of Control v. *Anaplasma* samples. Showing terms that are significantly enriched with an adjusted P-value of less than 0.05.

Other immune related genes were detected, including toll-like receptors *Tlr9* and *Tlr13*. These receptors detect bacterial nucleotide motifs, such as specific unmethylated DNA sequences or the 23s ribosomal RNA, respectively.

Table 7. Expression of genes associated with the NF-κB and JAK/STAT signaling pathways as found during infection with *A. phagocytophilum* in the skin.

Gene	Description	log2FoldChange	P-value
Trim30b	tripartite motifcontaining 30B	2.86	0
Trim30c	tripartite motifcontaining 30C	2.55	0
Stat2	signal transducer and activator of transcription 2	1.93	0
Trim30d	tripartite motifcontaining 30D	1.85	0
Stat1	signal transducer and activator of transcription 1	1.61	0
Ifi35	interferon-induced protein 35	1.51	0
Nmi	N-myc (and STAT) interactor	1.45	0
Il10	interleukin 10	1.43	0

Il10ra	interleukin 10 receptor, alpha	1.3	0
Trim12a	tripartite motifcontaining 12A	1.2	0
Stat4	signal transducer and activator of transcription 4	1.06	0
Card11	caspase recruitment domain family, member 11	1	0

Table 8. Expression of genes associated with cytoskeleton and the PI3K-Akt-mTOR signaling pathway as found during infection with *A. phagocytophilum* in the skin.

Gene	Description	log2FoldChange	P-value
Ptx3	pentraxin related gene	1.86	0
Aif1	allograft inflammatory factor 1	1.13	0
Plek	pleckstrin	1.02	0
Itga10	integrin, alpha 10	-1.32	0
Itgbl1	integrin, beta-like 1	-1.41	0
Ctgf	cellular	-1.64	0

	communication network factor 2		
Fgf14	fibroblast growth factor 14	-1.82	0
Myl3	myosin, light polypeptide 3	-3.3	0
Comp	cartilage oligomeric matrix protein	-4.04	0
Chad	chondroadherin	-4.8	0

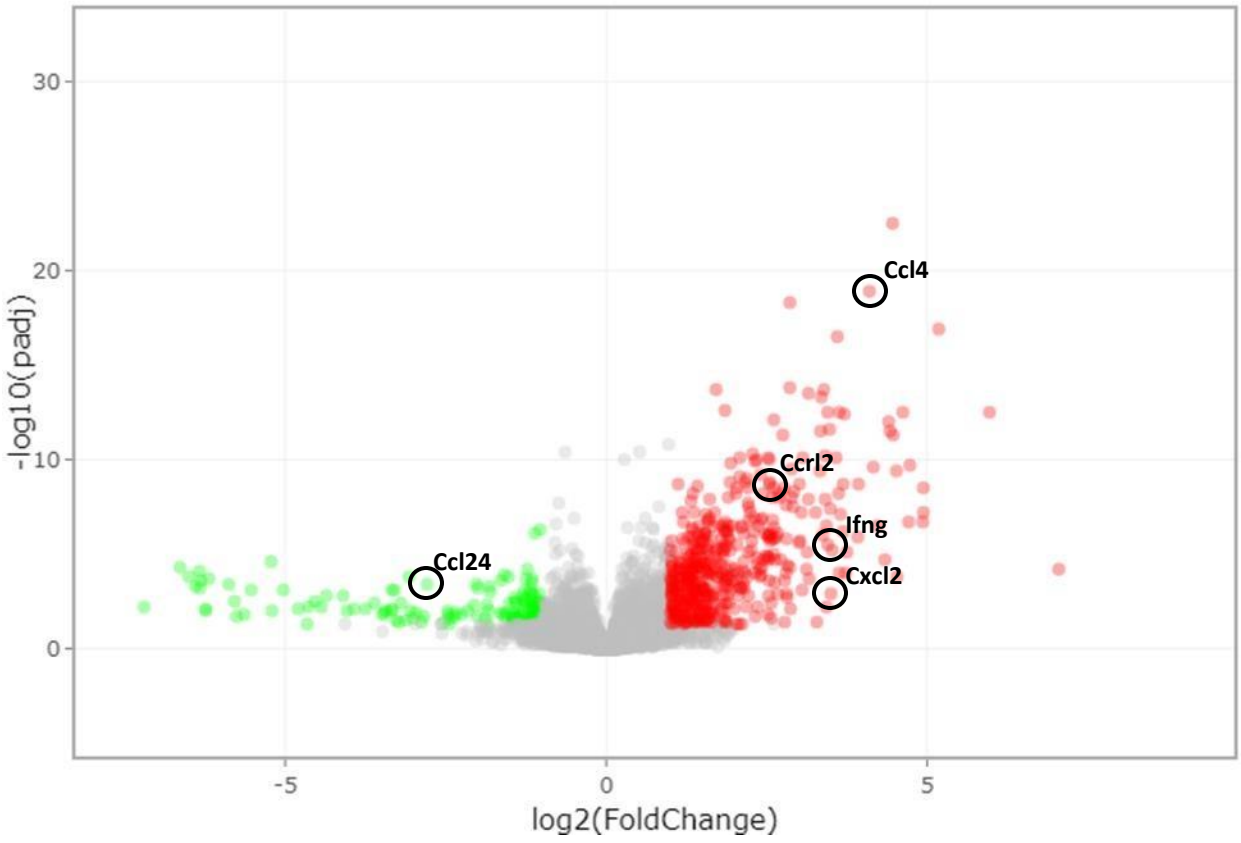

Figure 13. Volcano plot displaying upregulated (red) and down-regulated (green) genes 3 days into feeding from a nymphal *I. scapularis* tick infected with *A. phagocytophilum.* Select genes are marked on the plot, showing that many immune related genes are upregulated during infection (including Ccl4, Ccrl2, Cxcl2, and Ifng), while the only immune gene downregulated is Ccl24.

3.5 Infection in Ticks Affects the Secretion of Vesicles in Midguts and Salivary Glands

Ticks collected from the biopsies at the bite site were dissected and their salivary glands and midguts removed. Vesicles were purified from the media and quantified. *A. phagocytophilum* and *B. burgdorferi* infection led to the reduction of vesicles produced by both the midguts and salivary glands for *A. phagocytophilum*, but only a reduction in midgut vesicles for *B. burgdorferi*. Midgut vesicles 50-150 nm in size were significantly reduced during *A.*

phagocytophilum infection (P-value 0.0382; Figure 14A). Salivary gland vesicles 50-250 nm in size were also significantly reduced (P-values <0.0001, Figure 14B). For *B. burgdorferi* infections, midgut vesicles 50-150 nm in size were decreased (P-value <0.0001, Figure 14D). Salivary gland vesicles during *B. burgdorferi* infection produced no significant change, but with a trend of increased size (Figure 14C). In conclusion, tick-borne bacterial infection in ticks lead to physiological changes that alter to populations of extracellular vesicles produced in both the salivary glands and midgut, to a greater or lesser extent dependent upon the pathogen involved.

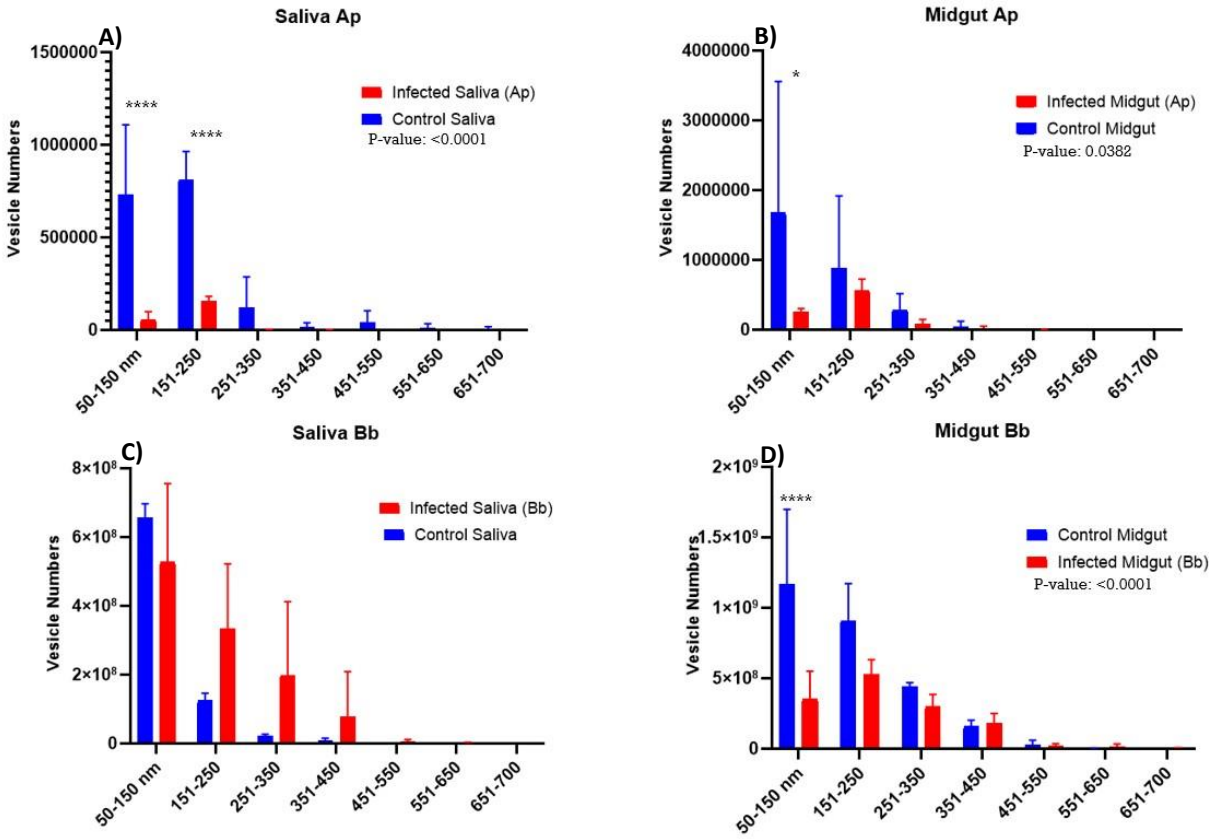

Figure 14. Populations of midgut and salivary vesicles during infection with *A. phagocytophilum* and *B. burgdorferi.* Vesicles 50-150nm in size were significantly reduced in the midgut by both pathogens.

3.6 Infection in Ticks Affects the Production of Cytokines and Chemokines

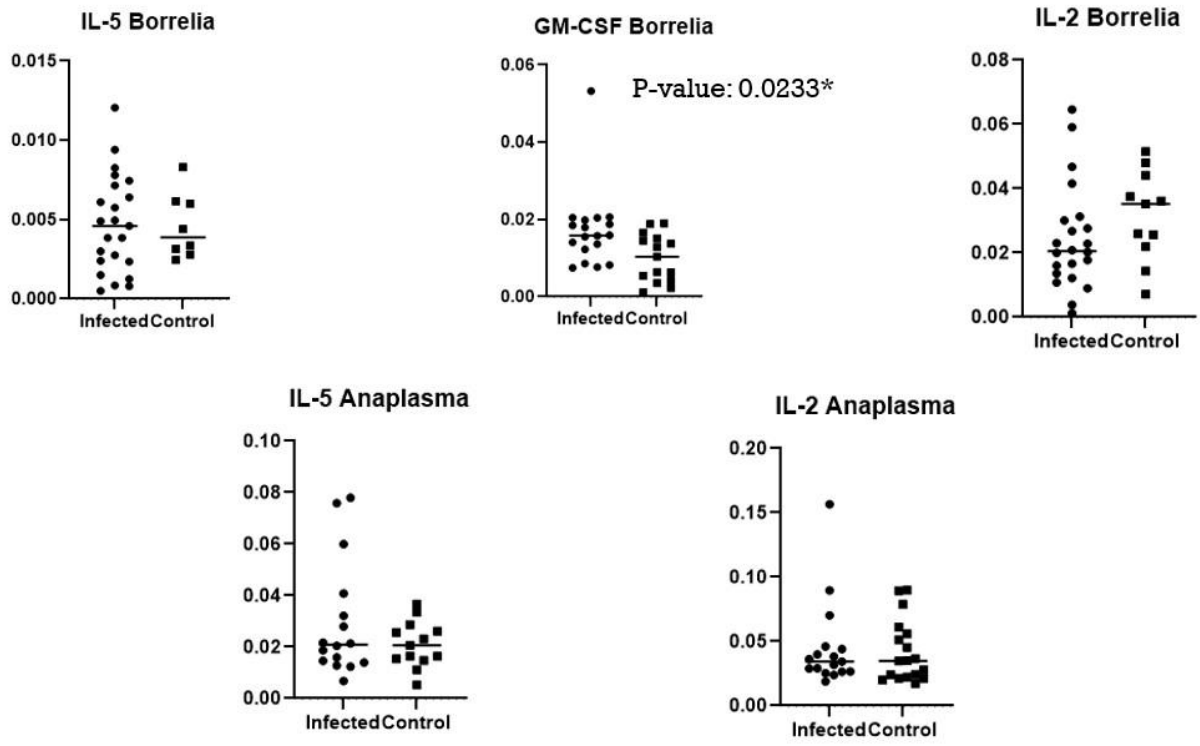

Figure 15. Tables displaying the levels of cytokines detected in the skin using sandwich ELISA. Values shown are the change in picograms across samples.

Collected skin samples were also homogenized and processed through ELISA analysis to quantify the amounts of cytokines and chemokines present at the bite site. In ticks infected with *B. burgdorferi*, the cytokines and chemokines analyzed were IL-5, GM-CSF, and IL-2 (Figure 15). Only GM-CSF showed a statistically significant change between infected and uninfected ticks. For ticks infected with *A. phagocytophilum*, the cytokines IL-5 and IL-2 were analyzed, with no significant change detected between infected and uninfected ticks.

4. Discussion

The effects of early *A. phagocytophilum* infection, particularly the events occurring in the skin at the site of transmission, are poorly understood. To complicate the issue of defining what events occur in the skin at the site of infection, the bite of an uninfected tick activates specific immune responses independently of the presence of bacteria. To discriminate and define the responses induced by tick feeding, the gene expression of non-damaged mouse skin was compared against the transcriptional profiles of skin at the bite site of an uninfected tick and the bite site of a tick infected with *A. phagocytophilum*.

Systemic infection with *A. phagocytophilum* and other intracellular pathogens leads to the increased production of IFN-γ and other proinflammatory cytokines and cellular responses [144, 167, 168]. IFN-γ is a powerful immune effector, modulating antigen presentation, anti-viral responses, leukocyte trafficking, and cell proliferation and apoptosis [169]. During feeding by ticks, we observed an upregulation of *Ifng*, the gene encoding IFN-γ. Multiple genes associated with IFN-γ were also upregulated. These results contrast previous observations of reduced IFN-γ in human neutrophils [170], but it is consistent with the systemic respond that occurs during infection in both human and equine models [171]. Unobserved during uninfected ticks feeding, ticks infected with *A. phagocytophilum* triggered an upregulation of *stat1*, another gene associated with IFN-γ mediated signaling. It is thought that IFN-γ activated pathways act as a priming method for host cells, enabling a faster and stronger response to other immune factors. The INF-II pathway involves IFN-γ binding to its receptor on the target cell, activating a signaling cascade that alters the transcription of targeted genes known as IFN- stimulated genes. The process begins with IFN-γ binding to the target cell's IFN-γ receptor, phosphorylating Janus kinase (JAK). JAK then associates with the IFN-γ receptor, allowing STAT1 to homodimerize

and become a γ-activated factor (GAF) that binds to a γ-activated sequences (GAS) located throughout the genome, activating transcription [172-174]. Curiously, the *Mus musculus* homologue of SOCS1 (suppressor of cytokine signaling 1) was upregulated during feeding by *A. phagocytophilum* infected ticks. The *Mus musculus* homologue of SOCS3 was upregulated during uninfected tick feeding. SOCS1 is a member of a protein family that acts as one of the central regulators of induced cytokine signaling, shown to be negative feedback loop that inhibits STAT1 and IFN-γ receptor activity through direct inhibition of JAK activity [174, 175]. This could mean that some subversion by *A. phagocytophilum* of the IFN-γ based defense response is occurring, since it is known that *A. phagocytophilum* infection results in changes to its host cells NF-κB and JAK/STAT signaling pathways. This process involves SOCS genes, but so too does natural control mechanisms in the host to prevent overexpression of proinflammatory cytokines [92, 173, 177, 178][176, 177].

In this study, genes related to the NF-κB and JAK/STAT signaling pathways were identified (Table 7). At this time, it should be noted that different cell types have alternate methods of dealing with infections, and the differences observed here may be due to the fact that skin samples represent a mixture of cell types, as opposed to an outlook focused on specific immune cell. It is possible multiple cell types crosstalk to overcome *A. phagocytophilum*'s immune evasion mechanisms in order to clear infection. Similarly, the timeframe of 3 days post-infection is likely to play some role on the differences in immune responses that we observe.

Observations show that TLR2 is the primary activator of NF-κB in mouse macrophages, with expression of *Tlr2* being upregulated when compared to the baseline [178]. Curiously, between control and *A. phagocytophilum* infected ticks, there was no change in *tlr2* or *tlr4* expression. These two receptors share an evolutionary lineage and both respond to bacterial cells wall

components [179]. Despite *A. phagocytophilum* lacking a cell wall, a total of 3 genes associated with the TLR4 pathway were upregulated, including *Nos2*, *Ccl3*, and *cd180*, although this could be due to redundance across immune response pathways. Additionally, *Tlr13* was upregulated during feeding by uninfected ticks. These receptors are associated with detecting bacterial nucleic acid motifs. This receptor and *Tlr9* were upregulated during feeding of *A. phagocytophilum* ticks compared to uninfected ticks.

The chemokines and cytokines quantified in this study (GM-CSF, IL-5, and IL-2) showed no change between pathogen types, other than GM-CSF, which showed significant upregulation either through RNAseq or ELISA. This is consistent with other studies, where neither IL-2 or IL-5 was significantly upregulated. It should be noted that while IL-5 is not significantly upregulated, it is consistently elevated above controls. This elevation has been shown to be important, as suppression of both IL-4 and IL-5 decreases spirochete populations [180]. GMCSF showed some elevation. Given that GM-CSF is a macrophage chemoattractant that also appears to stimulate a phenomenon known as coiling-phagocytosis of *B. burgdorferi* in monocytes, neutrophils, and eosinophils *in vitro* [181], indicating a protective response at the bite site.

A. phagocytophilum has been shown to alter the cytoskeleton of the cells it infects, likely to alter host cell physiology to its benefit. These alterations in the cytoskeleton can results in changes of gene expression, division, mobility, and perhaps most important to *A. phagocytophilum*, vesicular trafficking [182-185]. Genes related to the cytoskeleton were found to be both upregulated and downregulated in this study, likely meaning that *A. phagocytophilum* preferentially targets certain aspects of the cytoskeleton (Table 4). Interestingly, the downregulated genes are related to the PI3K-Akt-mTOR signaling pathway, where actin filaments are anchored to transmembrane receptors of integrins, proteins key to the formation of

focal adhesions where cells interact with the extracellular matrix [186]. During feeding by an uninfected tick, the PI3K-Akt-mTOR signaling pathway shows less differential expression, with only 6 associated genes having alterations in regulation. Of these 6 genes, 4 were upregulated. In the case of *A. phagocytophilum* transmission, of the 10 genes identified, only 3 were upregulated. This further supports the hypothesis that *A. phagocytophilum* could be modulating this pathway in the skin. Interestingly, most of the genes that were observed as downregulated in the skin were related to the extracellular matrix. This pattern of downregulation could imply modulation by *A. phagocytophilum* is occurring, but it is also known that immune cells and inflammation can restructure the matrix on their own [187]. This could mean the observations are not due to *A. phagocytophilum* directly, but are instead a response by host to *A. phagocytophilum* transmission. Restructuring of the extracellular matrix could delay in the repair of cells at the bite site, which may benefit *A. phagocytophilum* by creating a continuous recruitment of neutrophils for it to infect. Three genes associated with neutrophil chemotaxis were upregulated compared to feeding by an uninfected tick: *Cxcl3* (log2FoldChange: 2.32), *Trem3* (triggering receptor expressed on myeloid cells 3; log2FoldChange: 2.1), and *Csf3r* (log2FoldChange: 1.2). Numerous pathogens across different taxa are known to interact with the extracellular matrix, with its composition and restructuring being essential to pathogenesis. Remodeling of the extracellular matrix can promote motility and migration, as well as alter immune cell recruitment. Examples include *Leishmania*, *Leptospira*, *Candida*, *Aspergillus*, and *Trypanosoma* [188-190]. In this study it was observed that essentially all downregulated genes were genes associated with structural proteins, particularly those involved with the extracellular matrix. The upregulation of *A630012P03Rik* (Baseline-log2 fold change: 6.89; Control-log2 fold change: 4.93; P-value 0), a *Mus musculus* gene with no human orthologs, was observed highly

upregulated during *A. phagocytophilum* infection. This gene has been highly correlated to grooming behavior in mice, but the exact function of the protein encoded by this gene is unknown [191]. This gene may affect transmission by prompting the removal of tick vectors, preventing immunomodulatory salivary secretions to facilitate infection, or may reduce grooming to enhance it. This may be an example of defense mechanism against infection in the mice, focusing on a mechanical behavior-based response rather than immunological response to deal with infection. It could also be the result of *A. phagocytophilum* infection of the tick, since there is a significant reduction of salivary exosomes observed during infection. These exosomes may contain an effector designed to to reduce grooming is another possibility and the reduction of exosomes may result in upregulation of this gene.

In this study it was observed that extracellular vesicles from both the *I. scapularis* midgut and salivary glands were significantly reduced, particularly exosomes 50-150 nm in size, during bacterial infection. This was true for *A. phogocytophilum* infection, however infection with *B. burgdorferi* resulted only in a reduction in midgut vesicles. Salivary vesicles remained statistically unchanged. *A. phogocytophilum* could be sequestering lipids that would otherwise be used for production of vesicles for the creation of its own membranes as it grows and replicates inside midgut and salivary tissues. It could also be a result of restructuring of the tick microbiome, as this change could result in lowered symbiont populations, symbiont stress, or other factors that reduce the production of extracellular vesicles in the tick midgut [192]. Salivary glands could also be affected by microbiome restructuring, though to a lesser extent. It is known that *A. phogocytophilum* modulates tick salivary glands, and of these effects could potentially result in “specific cargo” for these cells, altering the types of vesicles produced by these cells [193, 194].

For *B. burgdorferi* the methods of modulation are unclear. It is known that the midgut microbiome is affected by *B. burgdorferi*, but in a much different way than *A. phogocytophilum*. *B. burgdorferi* is stimulated to multiply and feed in response to a bloodmeal, but many remain in the midgut despite the need to disseminate to the salivary glands for infection [195, 196].

5. Summary and Conclusion

5.1 Summary

In this study, I described the process undertaken to determine the changes that in gene expression and cytokine profiles during transmission of *A. phagocytophilum* and contrast it with the cytokine profiles during *B. burgdorferi* transmission. Additionally, the quantities of select cytokines in the skin and populations of extracellular vesicles produced by tick midguts and salivary glands were quantified. To decipher the events that take place at the bite site during *A. phagocytophilum* transmission, I performed an analysis of the *Mus musculus* skin transcriptome at the location of a tick bite site. Twenty-five *A. phagocytophilum* infected *I. scapularis* ticks were allowed to feed on mice for 3 days. After this time the mice were euthanized, and 3 mm skin punch biopsies were taken from the bite sites. The RNA from these samples was isolated and processed for RNAseq. As expected, the results identified the upregulation of several immune related genes, particularly those related to the IFN-γ, NF-κB and JAK/STAT signaling pathways. Multiple cytokines and chemokines were also upregulated with several results differing from previous studies with this organism, but differences in methods of analysis, species, and cell type may account for this. This suggests that whole system interactions are

important to the progression of disease, and further study is warranted to elucidate the entire picture.

Saliva is the primary way that ticks interact with their host. They use it to modulate their host's immune system to facilitate their own feeding, which can take weeks to complete. This saliva is known to contain extracellular vesicles known to transport the effectors sed to modulate the immune response of the host. To determine whether tick-borne pathogens modify the secretion extracellular vesicles from ticks *in vivo*, partially engorged *A. phagocytophilum* or *B. burgdorferi* infected *I. scapularis* nymphs were collected and dissected. The midguts and salivary glands from these ticks were excised and cultured in vesicle free media for 24 hours at 32°C to determine the numbers of vesicles produced. We observed that *A. phagocytophilum* significantly reduced the secretion of exosome size extracellular vesicles (50-150 nm in diameter). This effect is reduced in *B. burgdorferi* infected organs, with change limited to the midgut. The reduction of salivary vesicles could be resulting in the differential expression of immune genes observed during infection, as these pathogens are known to modulate tick saliva to facilitate transmission. Furthermore, the positive association between fitness and infection with *A. phagocytophilum* could mean that the tick could be altering its vesicles in a way that is beneficial to both organisms in some way.

5.2 Conclusion

A. phagocytophilum infection resulted in the upregulation of several immune pathways, including the IL2, TLR4, INF-γ, NF-κB, and JAK/STAT signaling pathways. While upregulation was observed, it was not across the whole pathway or, as in the case of TLR4, the receptor was not upregulated, only downstream associated genes. Downregulated genes primarily consisted of genes associated with the extracellular matrix, perhaps having something to do with cytoskeleton

reorganization and the PI3K-Akt-mTOR signaling pathway. The only downregulated cytokine was Ccl24, a known proinflammatory effector.

For populations of extracellular vesicles, for both midguts and salivary glands, infection with either *A. phagocytophilum* or *B. burgdorferi* results in a significant reduction in vesicles 50-150 nm in size. For *A. phagocytophilum* this reduction is more pronounced, with vesicles 151-250 nm in size also being significantly reduced in salivary glands.

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