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Characterization of Hemocytes in Amblyomma americanum and Investigation of TRAF6's Role in Proliferation of Hemocytes in Response to Infection

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Characterization of Hemocytes in *Amblyomma americanum* and Investigation of TRAF6's Role in Proliferation of Hemocytes in Response to Infection

By

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Characterization of Hemocytes in *Amblyomma americanum* and Investigation of TRAF6's Role in Proliferation of Hemocytes in Response to Infection

By

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Thesis Abstract

Amblyomma americanum is a medically important vector in the southeast United States. Cellular and molecular aspects of the immune system of this species were examined. To investigate cellular processes, hemocytes produced in response to gram-negative infection were characterized according to their ultrastructure. Four hemocyte types were identified: prohemocytes, plasmatocytes, type-I granulocytes, and type-II granulocytes. To elucidate molecular processes, we investigated AamTRAF as an immune-related gene by silencing the gene using RNAi then quantifying the hemocytes after inoculation with gram-negative and positive bacteria. It was found that silencing AamTRAF caused a decrease in hemocyte proliferation in response to gram-positive but not gram-negative bacteria. Knowledge gained from this research contributes to the understanding of the *Amblyomma americanum* immune system, with the long-term goal of determining how human pathogens evade detection in this vector.

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Introduction

Ticks are obligate blood-sucking ectoparasites classified as Acari order Parasitiformes (Walker et al., 2003). Ticks can be separated into two categories: hard and soft. The family name for hard ticks is *Ixodidae,* while soft ticks are known as *Argasidae*. Hard ticks are notably different due to their sclerotized dorsal scutal plate. There are approximately 850 species of ticks worldwide, with 90 of these residing in the United States. Ticks are a major vector of pathogens. Bacterial pathogens cannot be passed on transovarilly, instead they must be exchanged during a blood meal in between developmental periods (Sonenshine, 1991). Hard ticks are significant since they transmit the majority of medically important pathogens. These transmissions depend on the pathogen evading the tick immune system from one developmental period to the next. The tick immune system consists of hemolymph made up of hemocytes that secrete proteins and handle infections via phagocytosis, nodulation and encapsulation (Sonenshine, 1991). The ability of pathogens to elude these processes allows them to be passed on to tick's vertebrate hosts.

Medical Importance

Ticks are vectors for at least 16 different pathogens in the U.S., some of which

include *Anaplasma phagocytophilum*, *Borrelia burgdorferi*, *Babesia microti*, *Borrelia mayonii*, *Borrelia miyamotoi*, Bourbon virus, Colorado tick fever virus, Heartland virus, Powassan virus, and *Rickettsia parkeri* (CDC, 2019). These pathogens are responsible for tickborne diseases such as anaplasmosis, Lyme disease, babesiosis, ehrlichiosis, tularemia, spotted fever rickettsiosis, and Powassan virus encephalitis (CDC, 2019). From 2004 to 2016 these tick-borne diseases affected 491,671 people (Rosenberg et al., 2018). In the southwestern sphere of the United States, *Amblyomma americanum* is the most prevalent tick vector, transmitting at least six pathogens: *Ehrlichia chaffeensis, Ehrlichia ewingii, Francisella tularensis, Coxiella burnetii, Rickettsia amblyommii,* and *Borrelia lonestari.* The diseases resulting from the transmission of these pathogens cause a spectrum of symptoms that range from mild to severe and can even be fatal (CDC, 2019).

E. chaffeensis and *E. ewingii* are two closely related bacterial pathogens that are commonly found to be transmitted by *A. americanum*. These two cause similar symptoms making it difficult to differentiate their respective infections and diseases. Both cause symptoms such as fever, aches, and upset stomach. *E. chaffeensis* and *E. ewingii,* collectively, affect approximately 2,000 people every year (CDC, 2019). *F. tularensis* and *C. burnetii* are two other pathogens transmitted by *A. americanum*, along with other vectors of pathogens and disease. Both pathogens result in approximately 200 cases each year. Symptoms of *F. tularensis* include pneumonia, development of ulcers, tonsillitis,

and swelling. *C. burnetii* causes Q fever with symptoms such as fever, chills, fatigue, aches, and vomiting (CDC, 2019). Data, on the number of people affected by *R. amblyommii* and *B. lonestari* do not currently have data is not currently available by them. *R. amblyommii* causes common symptoms such as fever, headache, and rash. Symptoms of *B. lonestari* include the formation of a bullseye-shaped rash around the bite, fever, fatigue, muscle and joint pain, and headaches (CDC, 2019).

A. americanum has been implicated as the vector for two additional disease-causing pathogens: Heartland virus and Bourbon virus. These two tick-borne diseases are often characterized by a low white blood cell count. Heartland virus can cause a variety of symptoms that range from decreased appetite to muscle and joint pain. This virus has affected 50 people since June 2020 alone, with a majority of them being hospitalized; due specific methods to prevent or treat infection are not available. Bourbon virus was identified in 2014 and causes symptoms such as vomiting, body aches, rash, tiredness, and low platelet levels. Due to only recently being discovered, there is limited information on how many people are affected and long-term symptoms (CDC, 2019). The risk of becoming infected with these pathogens depends on whether you live in one of the areas in which *A. americanum* is present.

Population Distribution of A. americanum

A. americanum is present in 37 states within the U.S. (Eisen et al., 2016). Currently, populations of this species are highly condensed in the Central Midwest, with

predictions that they will expand into the Upper Midwest and further south due to climate changes (Raghavan et al., 2019). This is important because *A. americanum* is one of the species most frequently reported as being attached to humans in its range (Monzón et al., 2016). Continued expansion of ticks will lead to an increase in the vertebrate, specifically human, population exposed to them (Raghavan et al., 2019). Expansion of this species would lead to an increase in the number of people at risk of becoming infected with pathogens it transmits.

Transmission Cycle of A. americanum Pathogens

Various aspects of *A. americanum* biology make it an optimal vector for pathogens. *A. americanum* has four life stages: egg, larvae, nymph, and adult. Before advancement to nymph or adult stages a blood meal is required for molting. Additionally, *A. americanum* is a three-host species that feeds on a different host between each life stage. Pathogens can be obtained at the larval, nymphal, and adult stages during blood meals. During the molt required between each life stage, ticks retain their midgut and in doing so they retain pathogens picked up from the previous blood meal (Sonenshine, 1991).

The majority of the *A. americanum* life cycle consists of the time between hosts, which begins with eggs hatching and larvae attaching to a host. Larvae feed for five days on average before detaching from their host. They then molt their larval shell in approximately 22 days. To enter the nymphal stage and then proceed to find another host.

After feeding for five days, nymphs detach from the host and undergo a ~24-day nymphal molt. Females emerge and feed for ~12 days, when they will reach a point of engorgement and detach from host. After detachment, these females will undergo a gestation period of ~16 days, then oviposit eggs in a safe area, such as brush. The period from oviposition to hatching is referred to as incubation and takes on average 56 days for *A. americanum* (Troughton and Levin, 2007). Between each of these stages, a tick can live for up to a year without feeding; some individuals have been documented to live up to three years without a blood meal (Sonenshine, 1991). Taking all of that into consideration, pathogens can be harbored by ticks for years while evading the tick immune system.

Hemocyte Characterization

As previously mentioned, hemocytes play a large role in the tick immune response in ticks, making it important to understand the different types of hemocytes present and their purpose. Currently, hemocytes have not been characterized in *A. americanum*, but there have been seven different types of hemocytes identified in other tick species: prohemocytes, plasmatocytes, type-I granular hemocytes, type-II granular hemocytes, spherulocytes, adipohemocytes, and oenocytes. Although not all of these types are found in all species, some combination is present. Current literature shows that different tick species have their own combination of hemocyte types (Borovičková and Hypsa, 2005; Binnington et al., 1982; Brehe´lin and Zachary, 1986; Kuhn and Haug,1994; Fiorotti et al., 2019).

Different combinations of hemocyte types have been identified in *Ixodes ricinus*, *Rhipicephalus microplus*, and *O. moubata*. All three of these species have prohemocytes and some form of granular hemocytes. Both *I. ricinus* and *O. moubata* have type-II granular hemocytes, and *O. moubata* and *R. microplus* have spherulocytes. In *R. microplus* adipohemocytes and oenocytoids have been characterized (Kuhn and Haug, 1994; Feitosa et al., 2015). Each of these hemocyte types can be differentiated by their organelles and their role in the immune response.

Prohemocytes are hemocyte precursor cells found in *I. ricinus*, *O moubata*, and *R. microplus* that differentiate into all of the various hemocyte types ticks (Borovičková and Hypša, 2005; Kuhn and Haug, 1994; Zhioua et al., 1996). In ticks, these cells are generally round with a high nuclear to cytoplasmic ratio and have a homogenous cytoplasm rich with mitochondria. The majority of prohemocytes are found in the nymphal and larval stages of ticks and are uncommon in adults (Balashov et al., 1972). There is still limited information on prohemocyte differentiation in ticks. However, based on information from other arthropods it is believed that prohemocytes differentiate first into plasmatocytes. From there it is believed that plasmatocytes can then differentiate into one of three types of hemocytes: granulocytes, adipohemocytes, or oenocytoids. Oenocytoids can then further differentiate into spherulocytes (Hartenstein et al., 2006).

Plasmatocytes are hemocytes found in *I. ricinus* and *O. moubata* and are described as having Golgi bodies and lysosomes in various developmental stages (Borovičková and Hypša, 2005; Kuhn and Haug, 1994). These have been determined to

play a role in the phagocytic immune response via endocytic activity. It should be noted that endocytic activity was determined based on the abundance of lysosomal compartments in these cells (Kuhn and Haug, 1994). Endocytic activity by plasmatocytes takes place in both infected and uninfected ticks. When infected, these cells have been found to ingest and encapsulate pathogens (Hartenstein et al., 2006) and are thought to take part in the detection and lysosomal degradation of molecules and foreign organisms. In ticks, plasmatocytes are irregularly shaped. Additionally, their cell membranes feature small pits, or indentures, and vesicles attached to or within the membrane. (Kuhn and Haug, 1994).

Granulocytes are present in *I. ricinus, O. moubata,* and *R. microplus* and play an important role in the immune response via phagocytosis or encapsulation (Borovičková and Hypša, 2005; Kuhn and Haug, 1994; Fiorotti et al., 2019). There are two types of granulocytes, type-I and type-II. Type-I granulocytes are known to have a prominent rough ER with a few enlarged cisternae. They also have Golgi bodies associated with primary lysosomes and other vesicles. Spindle-shaped inclusions are present; these may be attached to ingested material or undergoing exocytosis (Borovičková and Hypša, 2005). Unlike type-II granulocytes, type-I have phagocytic granules and a low number of granula. These cells have many purposes, one of them being taking part in the coagulation of hemolymph after being wounded. In ticks, type-I granular hemocytes have been shown to display lysozyme-like immunoreactivity to bacteria, during which bacteria are broken down beginning with their cell walls (Khun and Haug, 1994). Type-II

granular hemocytes also display this lysozyme-like immunoreactivity to bacteria. These cells are differentiated from type-I by having ~100 spherical-shaped granula, these granula are electron-dense and in close proximity to the Golgi apparatus. It should be noted that in ticks, type-II granular hemocytes have three different developmental stages (Borovičková and Hypša, 2005). During the first developmental stage the Golgi apparatus is distinguishable and has few fully condensed granula, most of which are small. The second developmental stage shows larger granula in various stages of condensation. The last developmental stage is described to have a nucleus with lobes and no pseudopodia. Once matured, the rough ER can be seen clearly and, unlike in type-I, does not have enlarged cisternae (Borovičková and Hypša, 2005). Type-II granular hemocytes are also different from type-I in their immune response. Type-II granular hemocytes respond to infections via phagocytosis and encapsulation. Additionally, these cells are thought to be involved in non-immune functions such as storage, transport, and metabolization of glycogen during the blood meal (Kuhn et al., 1994; Borovickova et al., 2005; Hartenstein et al., 2006). Overall, both types of granulocytes play a large role in the immune response.

Lastly, spherulocytes, adipohemocytes, and oenocytoids are three hemocytes types that have been characterized (Borovičková and Hypsa, 2005; Fiorotti et al., 2019; Feitosa et al., 2015). Currently, their function in ticks is yet to be determined, but in other arthropods their functions are not immune-related. Spherulocytes are present in *O. moubata* and *R. microplus* and have membrane-bound granula, larger than those found in

granulocytes, with a tubular substructure. Adipohemocytes have been characterized in *R. microplus* and have been found to be irregularly shaped cells with lipid inclusions that fill the cytoplasmic space along with mitochondria (Borovičková and Hypsa, 2005; Fiorotti et al., 2019). Oenocytoids, irregularly shaped non-phagocytic hemocytes that play a role in encapsulation, are found in *R. microplus* (Fiorotti et al., 2019). In addition to being irregularly shaped, oenocytoids have an eccentric nucleus that is off-center in the cell, and homogenous cytoplasm along with small refractory granulations and mitochondria (Feitosa et al., 2015). It can be seen that from species to species of tick there can be differences in the hemocyte types present, making it important to identify which ones are present in *A. americanum*. Four of these hemocyte types have been shown to play an integral role in the first line of defense for several tick immune systems, making it imperative that we understand them in *A. americanum*.

Molecular Immune Response

Hemocytes respond in three ways to microorganisms: phagocytosis, nodulation, and encapsulation. Each of these processes works toward mitigating infection. For example, microorganisms that are phagocytosed are destroyed in a phagolysosome. This is the primary cellular defense when ticks encounter a microbial infection. Nodulation involves accumulating hemocytes to capture and degrade large amounts of bacteria. Parasites are targeted by encapsulation, which surrounds the intruder and destroys it via cytotoxic products or asphyxiation (Rosales, 2011). Based off of pathways resolved in

insects, there are three pathways by which ticks hemocytes are thought to be activated to respond to infection: the Janus kinase (Jak)-signaling transducer activator of transcription (STAT) pathway, the immune deficiency (Imd) pathway, and Toll pathway (Palmer and Jiggins, 2015).

The Jak-STAT pathway is known to be activated by gram-negative infections. The presence of gram-negative bacteria in the hemolymph results in the expression of a cytokine known as Upd3. Jak-STAT is then activated in the fat body which results in the subsequent movement of STAT to the nucleus (Agaisse et al., 2003). STAT then activates expression of immunity proteins such as TEP1. These immunity proteins then go on to fight the gram-negative infection (Palmer and Jiggins, 2015). In mosquitoes it is currently believed that this pathway results in promotion of phagocytic activity (Agaisse et al., 2003). Additionally, the Jak-STAT pathway participates in cross-talk with the Imd and Toll pathways.

The Imd pathway also plays an important role in response to gram-negative bacteria. When the pathogen associated molecular patterns (PAMPs) of these bacteria are recognized by pattern recognition receptors (PRRs) the Imd pathway is activated. This triggers a signaling cascade that ultimately cleaves Relish (a type of Nf-κB transcription factor) which translocates to the nucleus. Once there, it promotes other Nf-κB transcription factors which activate the JNK pathway and upregulates the production of antimicrobial peptides (AMPs) (Palmer and Jiggins, 2015). It should be noted that in

ticks and other chelicerates, the Imd pathway is highly reduced and lacks key components such as transmembrane peptidoglycan recognition proteins (PGRPs) and specific AMPs. Despite this, the Imd pathway is still functional (Chávez et al., 2017), implying that there is another receptor involved. It is important to note that while these transmembrane PGRPs are missing, there are still intracellular and extracellular PGRPs present (Palmer and Jiggins et al., 2015; Kurata, 2014). In insects there are two main types of PGRPs: short and long. Short PGRPs can be found in hemocytes but are mainly present in the hemolymph, while long PGRPs are mainly found in hemocytes (Dziarski and Gupta, 2006).

Unlike the Jak/STAT and Imd pathways, the Toll pathway responds to gram-positive infections. In many arthropods gram-positive bacteria are recognized by PRRs which then activate the Toll pathway. The cascade that results from the Toll pathway activates Nf-κB transcription factors. These transcription factors are then transported to the nucleus where they increase the expression of antimicrobial peptides (AMPs), which results in the upregulation of immune-related genes (Palmer and Jiggins, 2015). Despite their differences, one commonality between the Imd and Toll pathway is the presence of Tumor Necrosis Factor (TNF) Receptor-Associated Factor 6 (TRAF6), which precedes Nf-κB activation.

TRAF6

Currently, there is limited research on TRAF6 in ticks. *I. scapularis* is the only tick to have its full genome sequenced, through which the presence of TRAF6 has been confirmed in *A. americanum*. A study of *Haemaphysalis longicornis* found that a subtype of TRAF (not defined) affects both molecular and cellular immunity in ticks and that expression of a TRAF gene increased temporarily during immune defense against pathogens (Takechi et al., 2016). It should be noted that the *H. longicornis* sequence referred to here could not be found in GenBank.

While TRAF6 has not been functionally characterized in ticks, there is limited data on it's role in *Drosophila melanogaster*. Within this species, TRAF6 mostly regulates signaling through Toll and receptors homologous to it (Grech et al., 2000). TRAF6, along with other variant proteins, can be characterized by a C-terminal TRAF domain made up of an N-terminal coiled-coil region, along with a C-terminal β-sandwich (Wajant et al., 2001). TRAF6's protein domain in *D. melanogastor* has two zinc-finger domains and a RING domain (Grech et al., 2000). When bacteria are recognized by a PRR, such as a Toll-like receptor (TLR), an intracellular signaling cascade is activated. This cascade, which results in activation of Nf-κB transcription factors, involves a plethora of adaptor proteins including TRAF6, Pelle, and Dorsal. TRAF6 is known to physically and functionally interact with Pelle, and in doing so synergistically activate Dorsal. This signaling cascade is known to regulate hemocyte responses such as

proliferation, differentiation, activation, and autophagy (Hacker et al., 2011; Into et al., 2012; Yu and Levine 2011).

Pathogens have evolved ways to escape the immune system using processes, such as disrupting the formation of TRAF signaling complexes. Pathogens inhibit the effects of TRAF by undergoing ubiquitination, which results in the loss in production of RIG-1 and TLR signaling along with the inhibition of Type I interferon (IFN) production (Keating and Bowie, 2009; Jaing and Tang, 2010). In other cases, viral proteins take advantage of TRAF signaling to promote their own proliferation. One example of this is when the Epstein-Barr virus takes over TRAF6 to mimic signaling done by CD40 (Brown et al., 2001). Lastly, viruses will increase the expression of specific miRNAs that target TRAF (Paik et al., 2011). The fact that these pathogens have evolved to make changes, disrupt, or avoid TRAF shows the importance of TRAF to the immune system.

Objectives

Objective 1: Characterization of Hemocytes Responding to Bacterial Infection in *A. americanum*

There are few papers characterizing hemocytes in ticks, and none characterizing *A. americanum* hemocytes. Additionally, the papers that do exist have revealed that different species have different combinations of hemocytes. By characterizing hemocytes in *A. americanum* we can begin to understand why they are such competent vectors. In

this objective, ticks will be experimentally infected with gram-negative bacteria followed by hemocyte extraction; hemocytes will be analyzed by transmission electron microscopy.

Objective 2: Characterizing the role of TRAF6 in hemocyte response to infection using RNAi.

Previous studies in *D. melanogaster* have characterized TRAF6 as being involved in hemocyte activation. There are few papers characterizing the stimulation of hemocytes in ticks, and none characterizing it in *A. americanum* specifically. Therefore, in this objective TRAF6-like in *A. americanum* will be silenced and the effects of silencing during infection with both gram-negative and gram-positive bacteria will be assessed.

CHAPTER 1: CHARACTERIZATION OF HEMOCYTES IN *AMBLYOMMA AMERICANUM* POST-INFECTION WITH GRAM-NEGATIVE BACTERIA

ABSTRACT

Hemocytes of the hard tick *Amblyomma americanum* were characterized on the basis of their ultrastructure by transmission electron microscopy. Distinctions between hemocyte types were made according to presence or absence of granula, granular structure, lysosomal compartments, nucleus to cytoplasmic ratio and similarity to other hemocyte types defined among hard ticks. Three general types of hemocytes were identified including prohemocytes, plasmatocytes and granulocytes. Additionally, two subtypes of granulocytes were characterized, type-I and type-II granulocytes.

INTRODUCTION

Ticks are obligate blood-sucking ectoparasites that are vectors of at least 16 different pathogens in the Unites States (CDC, 2019). Among them is *Amblyomma americanum*, a prominent, and expanding, vector in the midwestern, southeastern, and Atlantic regions (Madison-Antenucci et al., 2020). *A. americanum* transmits 5 different medically-important pathogens including *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Francisella tularensis*, *Borrelia lonestari,* along with the causative agent for southern tick associated rash illness (STARI) (CDC, 2019). Notably, all of these pathogens are

gram-negative bacteria. Ticks can harbor these pathogens for years, during which time the pathogens evade the tick immune system and are often passed on to a host (Sonenshine, 1991).

It is currently believed the immune system of arthropods is limited to an innate immune system composed of cellular and humoral responses (Lavine and Strand 2002; Sonenshine and Hynes 2008). The cellular responses are mediated via hemocytes which respond via phagocytosis, nodulation, encapsulation, coagulation, and production of immune-related molecules in order to mitigate infection. Each of these processes tend to have specific targets that they handle. For example, phagocytosis primarily responds to microbial infections by destroying microorganisms in a phagolysosome. Nodulation is primarily a response to bacteria and encapsulation is a primary response to parasites (Rosales, 2011). Phagocytosis in ticks has been investigated and was found to be key in the innate immune response. Phagocytosis is generally carried out by plasmatocytes and granulocytes, two of the main hemocyte types reported in the literature (Borovičková and Václav, 2005; Feitosa et al., 2015; Fiorotti et al., 2019; Inoue et al., 2001; Kuhn and Haug, 1994).

Within two families of ticks, Argasidae and Ixodidae, there are three specific types of hemocytes that have been consistently found: phagocytic plasmatocytes, granulocytes, and nonphagocytic granulocytes (Borovičková and Václav, 2005; Feitosa et al., 2015; Fiorotti et al., 2019; Inoue et al., 2001; Kuhn and Haug, 1994). Additional cells

have been discovered among Argasidae such as spherulocytes, adipohemocytes, and oenocytoids. All of these cells differentiate from prohemocytes, which are rarely found, presumably since they quickly differentiation in response to foreign substances (Kuhn and Haug et al., 1994). The only hard ticks to previously have their hemocytes characterized are *Rhipicephalus microplus*, *Rhipicephalus sanguineus, Ixodes scapularis* and *Ixodes ricinus*. Within *Rhipicephalus microplus, Rhipicephalus sanguineus, Ixodes scapularis* and *Ixodes ricinus* seven types of hemocytes have been described: prohemocytes, plasmatocytes, type-I and type-II granular hemocytes, spherulocytes, adipohemocytes and oenocytoids (Borovičková and Hypsa, 2005; Binnington et al., 1982; Brehe´lin and Zachary, 1986; Kuhn and Haug,1994). Currently, there are no studies characterizing hemocytes in *A. americanum*.

Characterizing hemocytes present during gram-negative infection in *A. americanum* is important for several reasons. Firstly, most studies have examined interactions between tick hosts and their respective pathogens. While this is important, it gives no baseline for how ticks interact with medically important disease-causing pathogens that they harbor and transmit. Thus, there is a gap in understanding of the differences between the two interactions and therefore how select human pathogens are able to evade the tick immune system. Additionally, hemocyte classification and terminology among arthropods is largely based on insect studies, due a lack of tick literature on this topic. Furthermore, a majority of the literature characterizes hemocytes in soft ticks, which are not as medically relevant as hard ticks such as *A. americanum*. As

stated above, current literature indicates that soft ticks have a wider variety of specific hemocytes than hard ticks.

The aim of this study was to characterize hemocytes in *A. americanum* exposed to a gram-negative bacteri using transmission electron microscopy (TEM) and compare the population of hemocytes in *A. americanum* to those in other ticks.

MATERIALS AND METHODS

Tick Infections and Collection of Hemolymph

Amblyomma americanum was purchased from the rearing facility at Oklahoma State University. In this experiment, hemolymph was pooled from both male and female ticks to optimize the chances of cell retention. For injections, ticks were immobilized ventral side up on standard laboratory tape under the EZ4W stereomicroscope (Leica Microsystems Inc, Buffalo Grove, IL). Ticks were injected with *Escherichia coli* in the membranous region between the idiosoma and the fourth coxa using a syringe fitted with a 33-gauge, 30˚ bevel needle (Hamilton Company, Reno, NV, USA). Bacteria were prepared using an overnight culture of *E. coli* in LB broth with ampicillin (1μL/ mL). After 12 h the bacteria were harvested by centrifugation and washed thrice in diH_2O. *E. coli* was then diluted to an OD_{600} = 0.01 using a NanoVue Plus spectrophotometer (Biochrom Ltd, Cambridge, U.K.).

Unpublished data in the Porter lab has shown that hemolymph proliferation peaks around 24 h. For this reason, hemocytes were extracted 24 h post-infection. For

hemolymph extraction, ticks were taped dorsal side down using standard laboratory tape under an EZ4W stereomicroscope (Leica Microsystems Inc, Buffalo Grove, IL). A razor blade was used to remove the first tarsus of the first (superior) leg. Pressure was applied to the dorsum of the tick right above the anal groove. Secreted hemolymph was collected using a glass-pulled Pasteur pipette (~1 µL for females and ~.5 µL for males) and pooled in a collection tube filled with 500 µL of 1X PBS to prevent lysis. Hemolymph from 106 *A. americanum* (56 female and 50 male) was extracted.

Transmission Electron Microscopy

Hemocytes collected in PBS were centrifuged using a Micromax RF centrifuge (Thermo Fischer Scientific, Waltham, MA) at 500 xg for 3 min at 4°C. After this, the PBS was decanted; hemocytes were fixed in 1:1 mixture of 5% glutaraldehyde and 0.1 M sodium cacodylate buffer (pH 7.2) overnight at 4°C. Cells were washed three times in 2.5% sucrose in 0.1 M buffer for 15 min. After the third wash, a 1:1 solution of sodium cacodylate buffer and 2% osmium tetroxide was added for 2 h at 4°C followed by a 5 min wash in diH_2O. Cells were then dehydrated in acetone, infiltrated with Spurrs resin, and embedded in fresh resin which was polymerized at 70°C overnight. Ultrathin sections were prepared on a Ultramicrotome (RMC MTX) with a diamond knife (DiATOME) at a thickness of 100 nm. Sections were collected on 300 mesh copper grids, stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope (FEI Tecnai12) operating at 80KeV.

Hemocyte Characterization

Hemocytes are typically characterized based on factors such as presence of granula, cell shape and pseudopodia, nuclear characteristics, size and shape of the Golgi apparatus, and lysosomal compartments (denoting endocytic activity). Cells in this investigation were screened for four morphological types of granula (1) mostly rounded, fully electron-dense; (2) immature showing small electron-lucent granula with a dense core; (3) less electron-dense granula that are round, oval, or spindle shaped with prominent tubular structures; and (4) prominent "spherules" with tubular structure. Granula were the primary structures used to differentiate between hemocyte types due to their striking differences. Endocytic activity was identified by the presence of lysosomal compartments. Five types of lysosomal organelles have been reported in *I. ricinus* and *O. moubata*: primary and secondary lysosomes, pinocytic vesicles, multivesicular bodies, and heterogeneous bodies of resorptive nature. Other characteristics which have been used to delineate hemocyte type are the appearance and pattern of the cytoplasm (homogeneous vs. heterogeneous) and the number of mitochondria (Borovičková and Václav, 2005).

RESULTS

Out of 50 characterized cells, four distinctly different types of hemocytes were observed in *A. americanum* exposed for 24 h to *E. coli*: prohemocytes, plasmatocytes, and granulocytes.

Prohemocytes Prohemocytes were infrequently observed and made up ~4% of the hemocytes characterized. The average cell diameter of prohemocytes was 16 microns. The nucleus was central and took up a majority of the cytoplasmic space. Nuclear shape was inconsistent with both irregular (Fig. 1A) and ovular (Fig. 1B) shapes observed in this study. Electron dense heterochromatin lining the interior of the nuclear membrane was prominent. The cytoplasm contained a few small granules and vesicles.

Plasmatocytes Plasmatocytes made up ~32% of the hemocytes observed (Fig. 2). These cells were oval-shaped and approximately 17 microns in diameter, with acentric nuclei, secondary lysosomes, and mitochondria. These cells had endocytic vesicles with ingested bacteria (Figs. 2A-B), consistent with their phagocytic role.

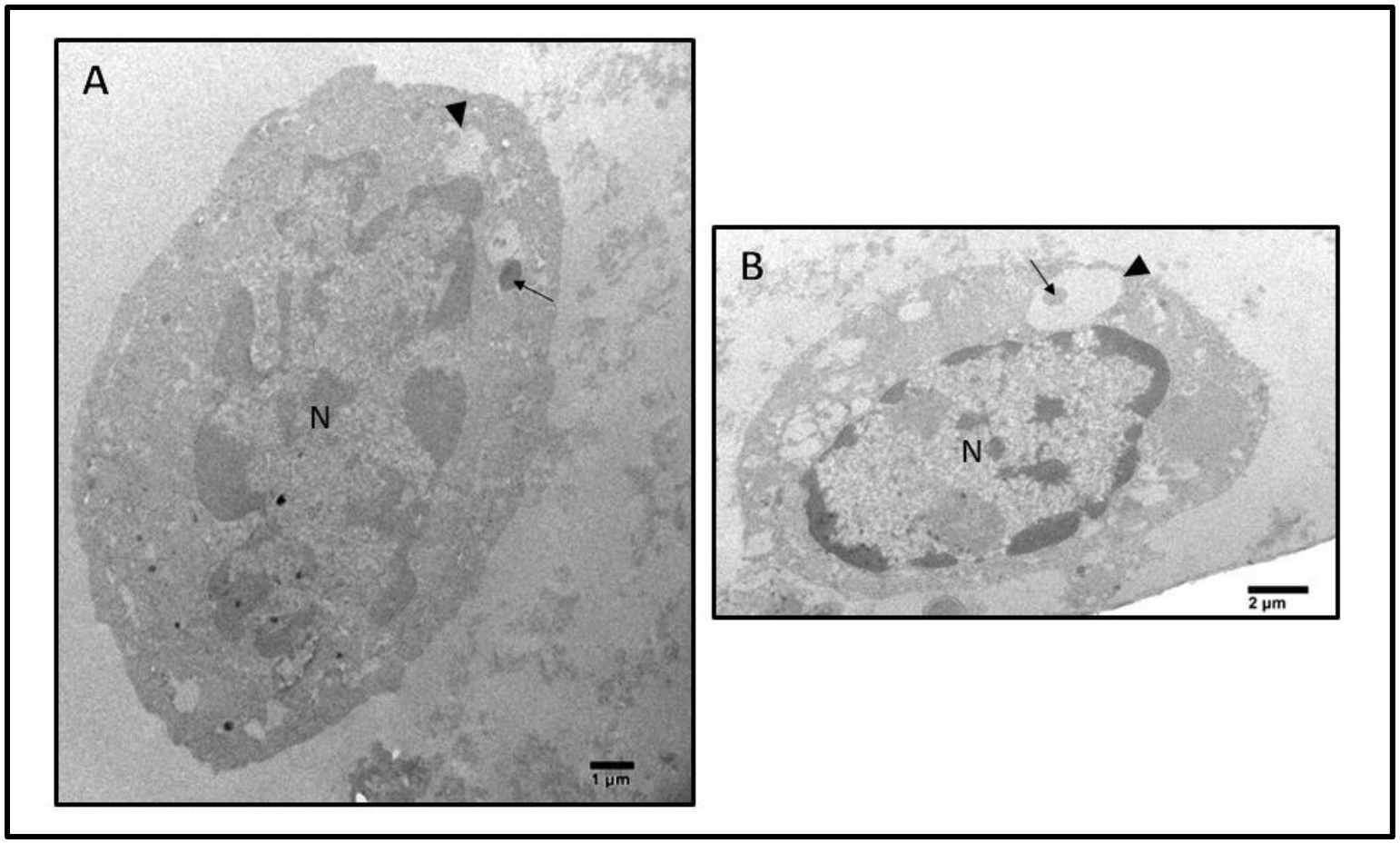

Figure 1: *Prohemocytes* **A)** An ovular cell with an irregularly shaped nucleus (N) that has a high nucleus/cytoplasm ratio. A granule (arrow) and developing lysosomal compartment (arrowhead) are present. **B)** An ovular cell with a centric ovular nucleus and a high nucleus/cytoplasm ratio. A lysosomal compartment (arrowhead) that contains a granule (arrow).

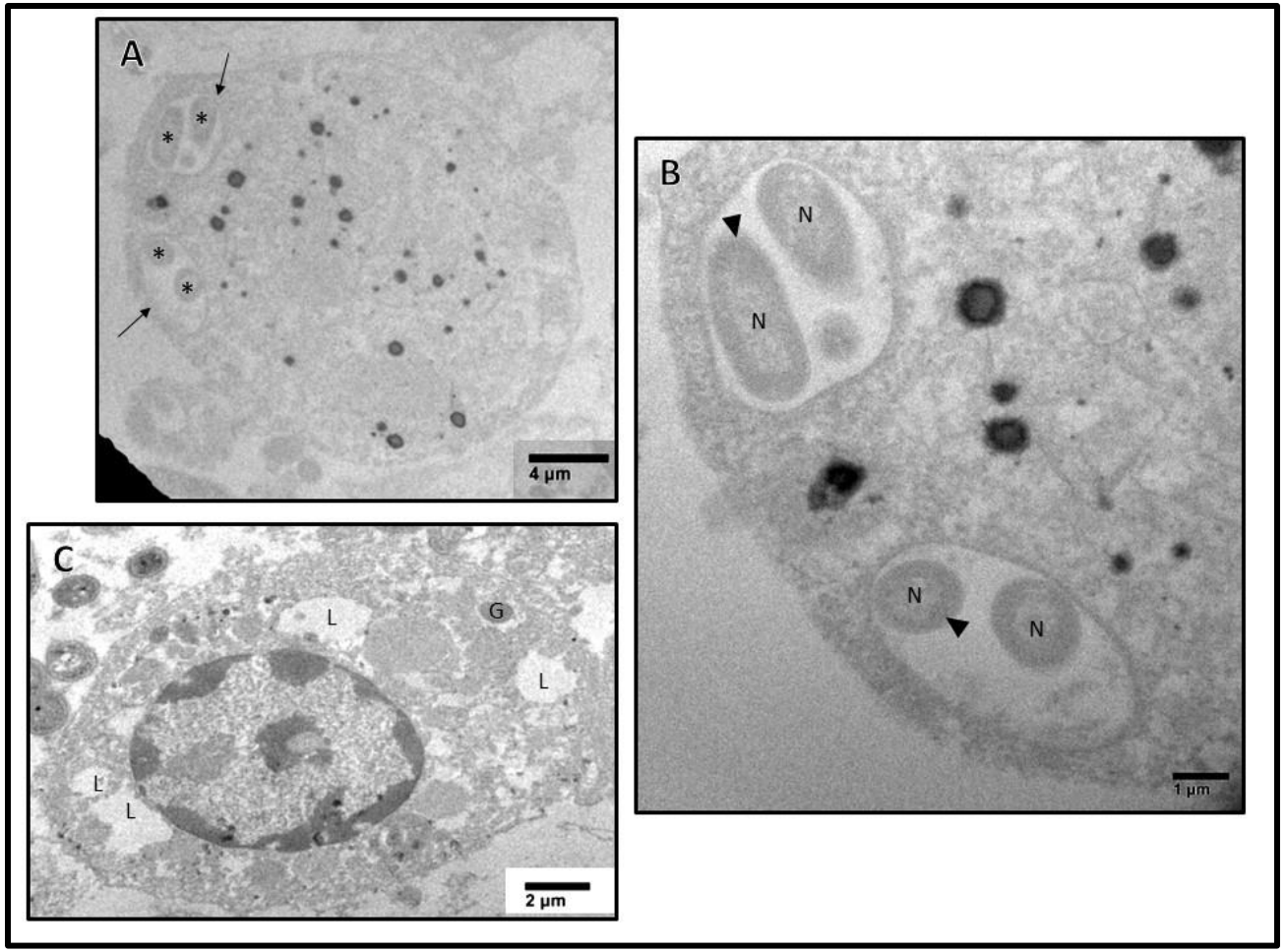

Figure 2: *Plasmatocytes*: **A)** An ovular cell with secondary lysosomes (arrows) containing bacteria (asterisks). **B)** Two lysosomal compartments containing bacteria whose nucleoid (N) and cell wall (arrowhead) can be identified. **C)** An ovular cell whose cytoplasmic space contains a few granules, some of which are contained within the lysosomes (G) but is mostly filled with lysosomal compartments (L).

Granulocytes More than 60% of the hemocytes observed were characterized as granulocytes (Figs. 3-4). These cells had variable shapes, ranging from elliptical to spherical, and an average diameter of 20 microns. Granula were prominent, numbering from 5- 30, and varying in developmental stage and electron density. Additionally, some of these cells contained vesicular regions within the cytoplasm. Specific features allowed for the identification of two granulocyte subtypes: type I and type II. Among granulocytes ~20% were type-I granulocytes and ~80% were type-II granulocytes.

Type-I granulocytes had centric nuclei and tended to have a low nucleus/cytoplasm ratio (Fig. 3B). In contrast with type-II granulocytes these cells had both electron-dense granula and some with inner tubular structures (Fig. 3A). Additionally, primary lysosomes were visible, which is one of the defining ultrastructural differences between type-I and type-II granulocytes.

Type-II granulocytes had acentric nuclei (Fig. 4A) and were averaged 21 microns in diameter. These cells tended to be circular and contained electron-dense granules of various shapes and sizes that filled the cytoplasmic space (Fig. 4). Some of the granules appeared to be undergoing condensation. These cells displayed no signs of endocytic activity. A few of these cells contained granules with rod-like, electron dense structures (Figs. 5).

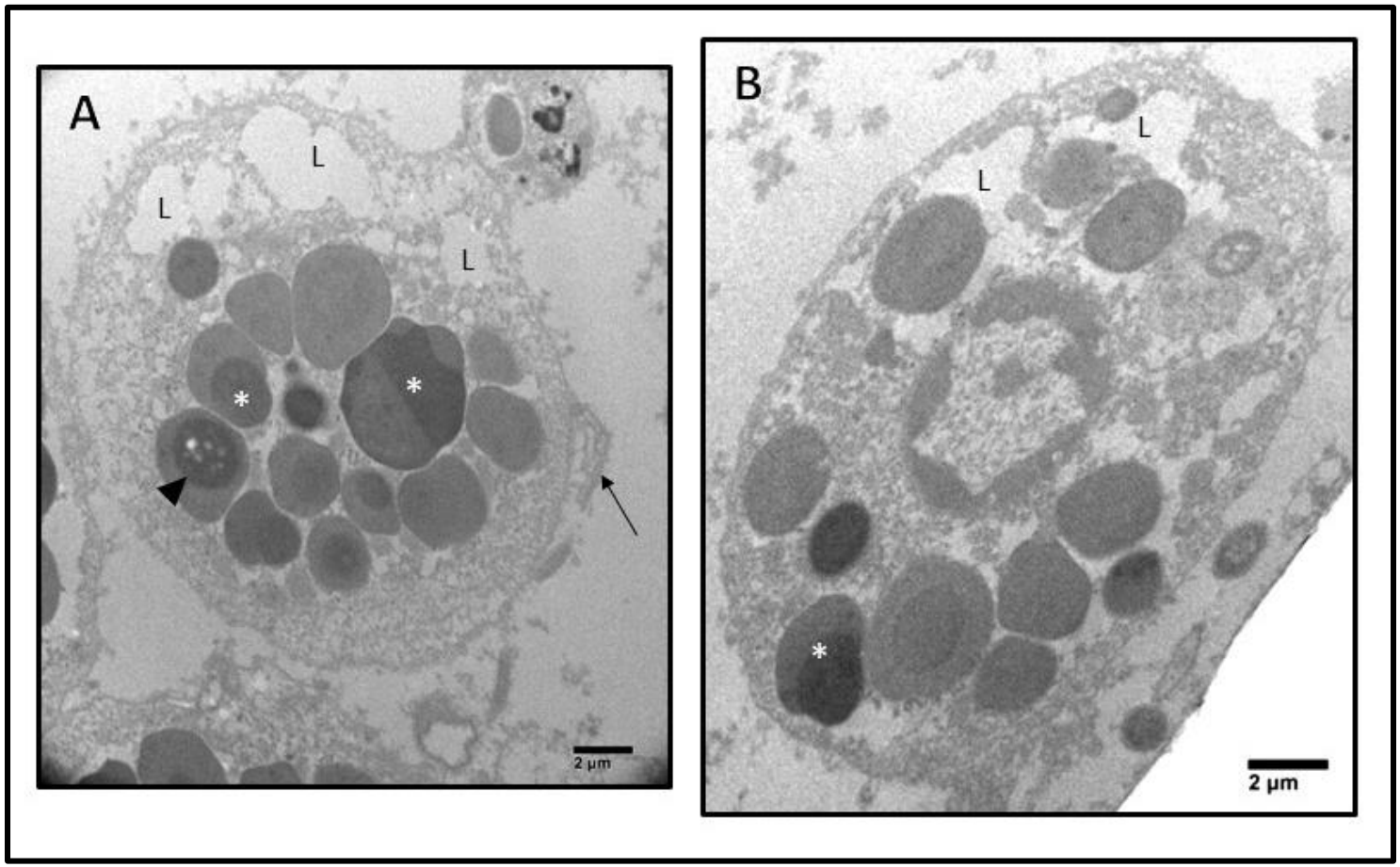

Figure 3: *Type-I Granulocytes* **A)** Circular granulocyte containing granules with tubular structure (arrowhead), condensing granules (asterisks), lysosomal compartments (L), and pseudopodia (arrow). **B)** Ovular granulocyte with granules in a variety of stages of condensation (asterisks) and lysosomal compartments.

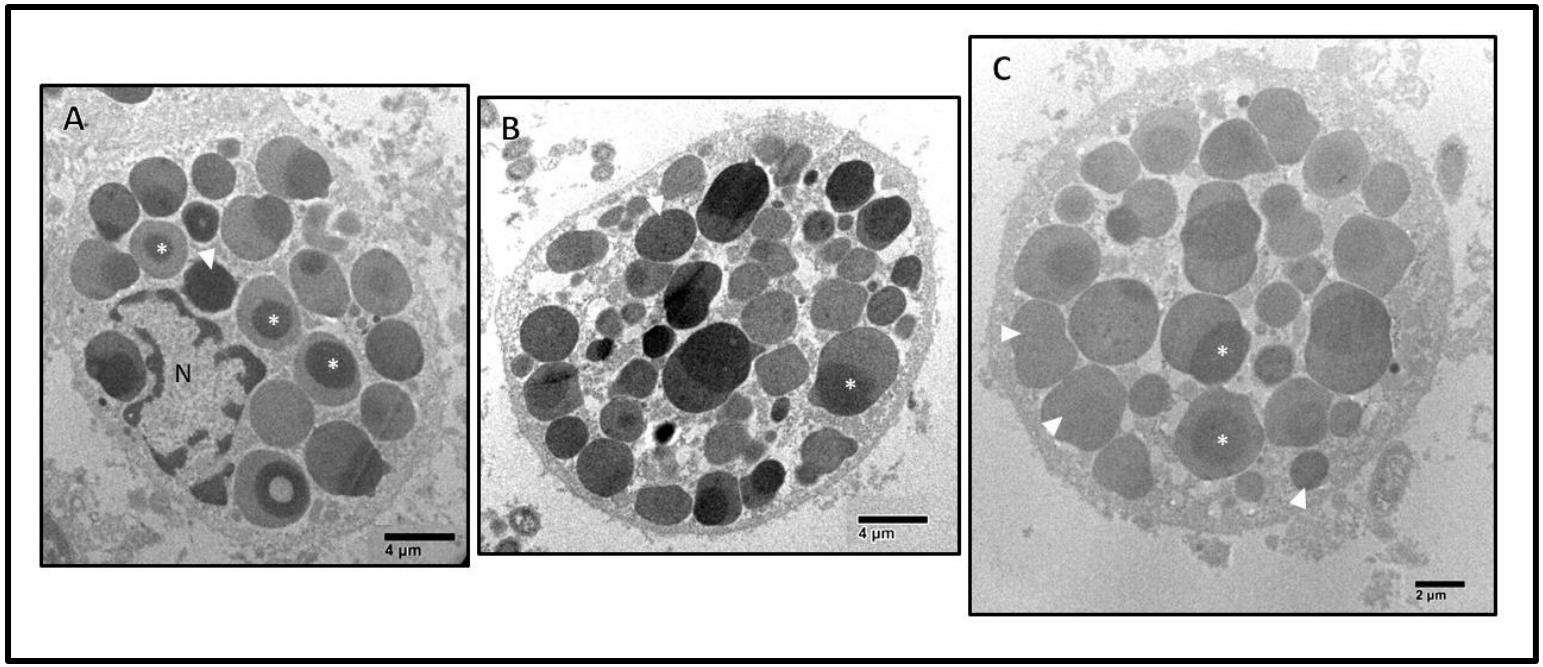

Figure 4: *Type-II Granulocytes* **A)** An ovular cell with an acentric irregularly shaped nucleus (N) and the cytoplasmic space filled with condensed granules (arrowhead) and condensing granules (asterisks). **B)** An ovular cell whose cytoplasmic space is completely filled with granules. A majority of these granules are electron dense and completely matured. **C)** A circular cell with granules filling the cytoplasmic space. Both condensed granules (arrowhead) and condensing granules (asterisks) are present.

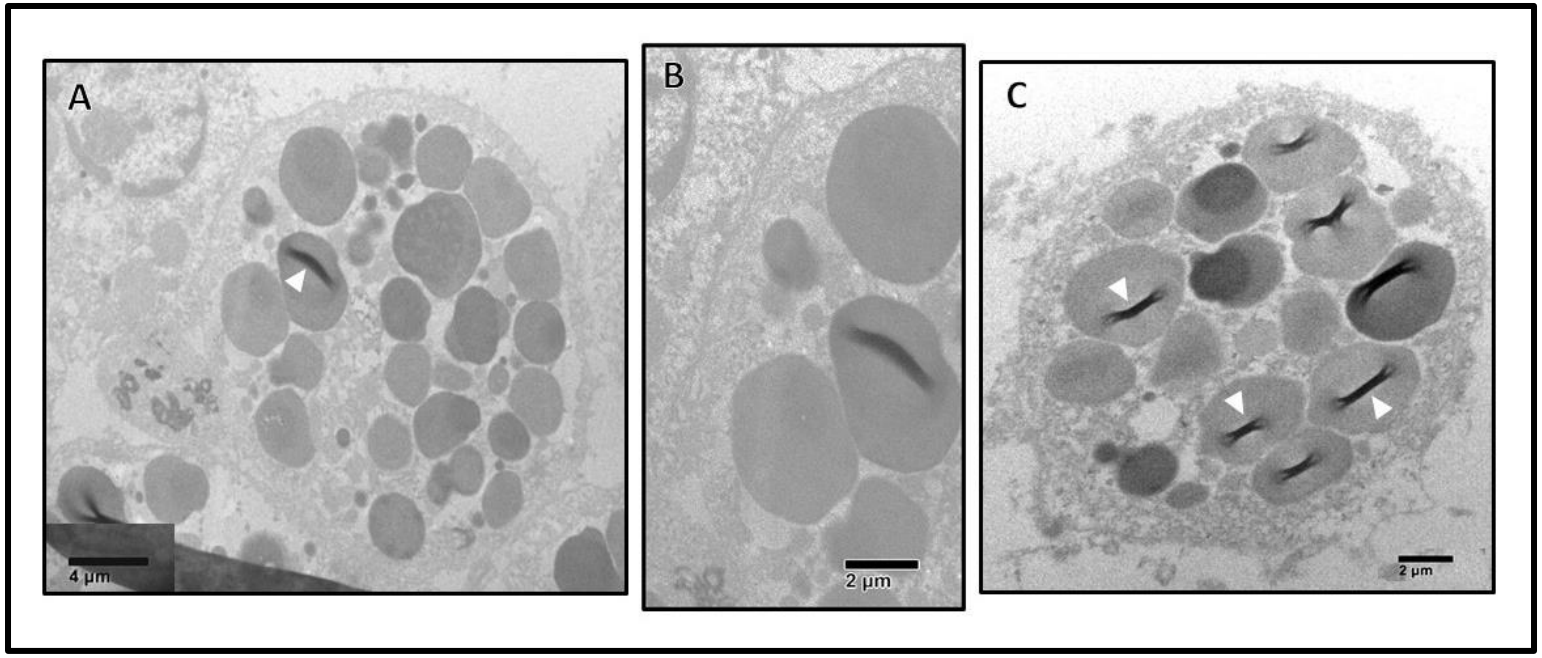

Figure 5: *Type-II Granulocytes with unique granule ultrastructure*. **A)** A typical type-II granulocyte with one granule filled with a long rod-like structure (arrowhead). **B)** A close up of the unique granule shown in 5A. **C)** A type-II granulocyte with multiple (7 out of 16) granules filled with rod-like structures (arrowheads)

DISCUSSION

The aim of this study was to characterize the hemocytes within the hard tick *A. americanum* on the basis of ultrastructure. Multiple authors have emphasized the importance of sampling and fixation techniques on the resulting ultrastructure of hemocytes (Borovičková and Hypsa., 2005; Binnington et al., 1982; Brehe´lin and Zachary, 1986; Kuhn and Haug,1994). Differences in fixation can result in methodical artifacts that compromise distinction between hemocyte types (Borovičková et al., 2005). While this is stressed, there is no consistent procedure for fixation of hemolymph among ticks or even arthropods. In this study, a procedure was developed in an attempt to minimize ultrastructural artifacts caused by sampling and fixation. This was performed by first collecting hemocytes in PBS in order to increase cell recovery and recreate an environment similar to hemolymph (pH 7.2) (de Paulo et al., 2018). Immediately after extraction was completed hemocytes were centrifuged and chemically fixed. The hemocytes prepared in this manner had ultrastructural characteristics consistent with literature reports from other tick species.

Hemocytes have been characterized in five other types of ticks, one being the soft tick *Ornithodorus moubata*. Four types of hemocytes have been distinguished in this species: prohemocytes, plasmatocytes, type-II granular hemocytes, and spherulocytes (Borovičková and Hypša, 2005; Inoue et al., 2001; Kadota et al., 2003). In addition to soft ticks, *Rhipicephalus microplus*, *Rhipicephalus sanguineus*, *Ixodes ricinus* and *Ixodes*

scapularis are currently the only other hard ticks to have their hemocytes characterized, with seven types of hemocytes described: prohemocytes, plasmatocytes, type-I and type-II granular hemocytes, spherulocytes, adipohemocytes and oenocytoids. In this paper, we will be comparing the ultrastructure of the aforementioned hemocytes (Kuhn and Haug, 1994; Feitosa et al., 2015; Fiorotti et al., 2019; Borovičková and Hypša, 2005) with those found in infected *A. americanum.*

Prohemocytes

Typically, a true prohemocyte would have undifferentiated cytoplasm. In this study, a prohemocyte with completely undifferentiated cytoplasm (i.e., lacking the complement of organelles associated with other hemocyte types) was not observed. Instead, prohemocytes had minimal differentiation of the cytoplasm, but possessed lysosomal compartments (Fig. 1). Notably, other studies in hard ticks have failed to find cells that could definitively be identified as prohemocytes due to organelles present in the cytoplasm. Khun and Huag (1994) defined similar cells as prohemocyte-like, partially due to the fact that during light microscopy they couldn't distinguish these cells from smaller plasmatocytes that didn't have prominent secondary lysosomes. Other researchers have reported that more prohemocytes are found in larvae and nymphs than in adult ixodid ticks (Balashov et al., 1972). Additionally, it has been proposed that there is a low probability of detecting prohemocytes due to rapid differentiation of the cytoplasm (Borovičková and Hypša, 2005), such as would occur during the peak infection period in which hemolymph was extracted in this study.

The ultrastructural characteristics of the prohemocytes observed in this study are consistent with those reported in prohemocytes found in other hard ticks (Borovičková and Hypša, 2005; Kuhn and Haug, 1994; Zhioua et al., 1996), including a high nucleus to cytoplasmic ratio and heterochromatin lining the nuclear membrane (Fig. 1). Nuclear shape in prohemocytes has been reported to vary. Both *I. ricinus* (Kuhn and Haug, 1994) and the *A. americanum* prohemocyte in Figure 1A have an irregularly shaped nucleus with electron-dense material lining the nuclear membrane. On the other hand, *I. scapularis* and the *A. americanum* hemocyte depicted in Figure 1B have oval-shaped nuclei. Nuclei are typically circular in shape, but it has been shown that when eukaryotic cells differentiate into specific cell types their nuclei can change shape (Dahl et al., 2008). This would explain why some prohemocytes were observed to have irregularly shaped nuclei, especially under infection conditions during which prohemocytes rapidly differentiate to minimize damage to the tick.

The prohemocytes observed in this study were likely developing into plasmatocytes or type-I granular hemocytes. This conclusion is based upon the lysosomal compartments present, as these two cell types are the only types of phagocytic hemocytes defined in the tick literature. No further conclusions can be drawn due to the early stage of maturation these cells were in.

Plasmatocytes

As previously stated, the two types of phagocytic hemocytes identified within ticks are plasmatocytes and type-I granulocytes. Both of these cell types are extremely similar in ultrastructure with the main differences being that the majority of the cytoplasmic space in plasmatocytes is filled with lysosomal compartments, including secondary lysosomes (Borovičková and Hypša, 2005). A disputed ultrastructural feature between these two cell types is the presence of small granula in plasmatocytes. Khun and Huag (1994) acknowledge that plasmatocytes do not have granular inclusions but state that intermediate forms were found. In Borovičková and Hypša (2005) *I. ricinus* plasmatocytes were shown to have no granular inclusions, while those of *O. moubata* did. It was noted that this discrepancy could be due to sections being collected from regions of the cell that were poor in granula. Additionally, other publications identify granula-poor or agranular cells to be plasmatocytes, possibly to align with the classification of hemocytes in insects (Borovičková and Hypša, 2005). In this study, plasmatocytes were defined as cells in which the cytoplasmic space was filled with lysosomal compartments and few to no granular inclusions (Fig. 2), consistent with the classification of Kuhn and Huag (1994) and Borovičková and Hypša (2005).

Granulocytes

Type-I granular hemocytes are similar to plasmatocytes except that they present granular inclusions (Borovičková and Hypša, 2005; Kuhn and Haug, 1994). According to the characterization of different developmental periods in type-I granular hemocytes

within *I. ricinus*, the type-I granular hemocytes observed in *A. americanum* exposed to *E. coli* for 24 h were identified as mature (Borovičková and Hypša, 2005; Kuhn and Haug, 1994). This conclusion is based on the lysosomal compartments observed within these cells (Fig. 3).

Type-II granular hemocytes, a type of nonphagocytic granular hemocyte, were also observed in *A. americanum* exposed to *E. coli* for 24 h. A defining characteristic of these cells is that dense granules take up a majority of the cytoplasmic space (Fig. 4). According to the stages of type-II granulocyte development defined by Borovičková and Hypša (2005), a majority of these cells in *A. americanum* were in their second developmental stage (Fig. 4). A key feature of this stage is that granula are in the process of condensing into mature granules, as indicated by the presence of electron-dense cores that do not extend to the end of the granula. Type-II granulocytes are considered to be mature when their granula present as consistently electron-dense, as described in *I. ricinus*, *I. scapularis*, and *O. moubata* (Borovičková and Hypša, 2005; Kuhn and Haug, 1994; Zhioua et al., 1996).

A few type-II granulocytes had unique rod-like structures within their granules (Fig. 5), similar in ultrastructural appearance to cationic proteins found in eosinophils of mammals (Cross and Mercer, 1993), but not previously reported in arthropods. These eosinophilic proteins have antimicrobial activity against both gram-negative and gram-positive bacteria (Torrent et al., 2008). Cytochemical methods should be employed in

future investigations to thoroughly characterize the electron-dense rods in these *A. americanum* type-II granulocytes.

Other Cell Types

Three hemocyte cell types defined in soft ticks were not found in *A. americanum* exposed to *E. coli*: spherulocytes, adipohemocytes, and oenocytoids. The functions of these cell types have yet to be determined in ticks, partially due to their rarity, but in other arthropods their functions are not immune-related. For example, within Lepidoptera, spherulocytes are believed to be involved in the production of mucopolysaccharides (silk) (Akai and Sato, 1973; Brehélin and Zachary, 1986). Within arthropods, adipohemocytes have not been separated as a distinct cell type due to their ultrastructural similarities to granulocytes, with the main difference being the presence of lipid droplets in the former (Falleiros et al., 2003). Within *Musca domestica*, adipohemocytes were considered to be a functional variant of other cell types with lipid droplets (Lea and Gilbert, 1966). Oenocytoids were found in the hemolymph of *Rhipicephalus microplus* prior to fungal infection, but not post-infection, indicating that these cells do not have an immune-related function within ticks (Fiorotti et al., 2019).

Conclusion

In this study, four specific types of hemocytes were characterized for the first time in *A. americanum*: prohemocytes, plasmatocytes, type-I granulocytes, and type-II granulocytes. The ultrastructural features of these cell types were consistent with reports

from other tick species, including *I. scapularis*, *I. ricinus*, and *O.moubata* (Borovičková and Hypša, 2005; Kuhn and Haug, 1994; Zhioua et al., 1996). This research documents the characterization of hemocytes during infection with a gram-negative bacterium in *A. americanum*. With an understanding of the *A. americanum* hemocytes stimulated by a bacterium that they are not a vector for, the opportunity to contrast this response to one directed against vertebrate pathogens that are transmitted by ticks now exists. Further research to fully characterize the populational and cytochemical responses of *A. americanum* hemocytes to both pathogens and nonpathogens is needed. In doing so, the mechanisms by which human pathogens are able to evade the tick immune system on the cellular level can be elucidated.

CHAPTER 2: SILENCING OF A TRAF GENE IN *AMBLYOMMA AMERICANUM* REDUCES HEMOCYTE PROLIFERATION IN GRAM-POSITIVE BUT NOT GRAM-NEGATIVE BACTERIAL INFECTION

ABSTRACT

Tumor necrosis factor receptor-associated factor (TRAF) proteins serve as immune system regulatory molecules across taxa. In invertebrates, TRAFs function in the immune response to both gram-negative and gram-positive bacteria. In this study, we identified a partial transcript annotated as a TRAF protein in *A. americanum.* Comparative sequence and phylogenetic analyses reveal strong sequence divergence from other TRAFs, notably in the RING domain region. To assess whether this protein serves an immune role, we performed RNAi and assessed the impact of silencing during gram-negative and gram-positive bacterial infections by quantifying the hemocyte proliferation response. Our results show no statistical difference between hemocyte proliferation in response to gram-negative bacterial infection in silenced and control ticks. However, for gram-positive infection, hemocyte proliferation was significantly reduced as compared to control ticks. These results suggest a role for *Aam*TRAF in the pathways that recognize and respond to gram-positive bacteria, such as the Toll pathway.

Data in this study contribute to the understanding of the differential immune response employed by ticks against these two types of bacteria.

INTRODUCTION

Ticks represent one of the most important vectors of disease-causing pathogens to both humans and animals throughout the world. In the United States, ticks are responsible for the vast majority of vector-borne disease cases (Rosenberg et al., 2018), with nine species primarily responsible for pathogen transmission to humans (CDC). Of these, *Amblyomma americanum* is one of the most important vectors in the southeastern, Atlantic, and midwestern regions (Madison-Antenucci et al., 2020). Notably, *A. americanum* transmits several bacterial pathogens: *Ehrlichia ewingii, Ehrlichia chaffeensis*, *Francisella tularensis*, and the causative agent for STARI (CDC). As with other vectors, transmitted bacterial species that cause disease are gram-negative (gram-) while there are no documented cases of biologically vectored disease-causing gram-positive (gram+) bacteria. The reasons for this have not been elucidated, although in general, the capacity for a tick to vector a particular pathogen is determined in part by their immune response or immune tolerance to these invading microorganisms (Alberdi et al., 2016; Martins et al., 2017; Tully et al.; 2020, Hajdušek et al., 2013; Park et al., 2021).

Studies investigating the tick immune response have grown significantly in recent years. Based on our current understanding, the tick immune response to bacteria

somewhat parallels that of insects in that it can be divided into the IMD and Toll pathways that are activated by gram- and gram+ bacteria, respectively, as well as the JAK/STAT pathway that is considered a branch-off of the IMD pathway and that participates in response to gram- infection (Olivia Chávez et al., 2017; Fogaça et al., 2021). The IMD pathway is activated by the recognition of DAP-peptidoglycan type pathogen-associated molecular patterns (PAMPs; Olivia Chávez et al., 2017; Fogaça et al., 2021). The Toll pathway is also activated by peptidoglycans, but of the lysine type (Olivia Chávez et al., 2017; Fogaça et al., 2021).

According to the modeled Toll pathway in ticks, the recognition of gram+ infection results in the activation of Spaetzel that binds to a toll-like receptor on the cell surface membrane, followed by recruitment of MyD88 that interacts with two additional adapter proteins known as Tube and Pelle, that then activate TRAF. These interactions lead to the activation of Dorsal via the inactivation of the Dorsal inhibitor known as Cactus. Dorsal is also an NF-κB transcription factor that translocates to the nucleus to induce the expression of anti-microbial genes (Olivia Chávez et al., 2017, Fogaça et al., 2021).

On the other hand, a TRAF in *Haemaphysalis longicornis*, H1TRAFD1, was characterized as responsive to gram- infection (Takechi et al., 2015). In other invertebrates, TRAF genes have been functionally characterized to participate in response to both bacterial types. In the Zhikong scallop, a TRAF6 homolog was found to be

downregulated in the presence of peptidoglycan (Qiu et al., 2011), while in the noble and yesso scallops and in the pearl oyster, TRAF genes were upregulated in gram- infection (Zhang et al., 2021; Wang et al., 2015; Huang et al., 2012). Still, other studies have found TRAFs functioning in anti-viral defense in invertebrates, including in ticks (Wang et al., 2011; Li et al., 2020; Lemasson et al., 2021). Results from these studies suggest that multiple types of TRAFs function in invertebrate response, potentially in a pathogen-specific manner.

In this study, we identified and characterized a partial TRAF transcript in *A. americanum*. Similarity with other tick TRAF sequences was relatively low. It was also found that the RING domain region differed from the canonical form. To determine whether this protein might play a role in bacterial immune response, we performed RNAi and assessed the impact of silencing on hemocyte proliferation as a measure of the tick's ability to control infection. Additionally, we tested the impact of silencing for both gram- and gram+ infection types. Our results showed that in comparing infection types in non-silenced ticks, there is no significant difference in the numbers of responding hemocytes at 24 hours after infection. However, there was a significant reduction in the ability to generate a hemocyte response for *Aam*TRAF -silenced *A. americanum* ticks in gram+ infection. These results suggest that TRAF could be a novel protein involved in detecting and responding to gram+ infection.

MATERIALS AND METHODS

Bioinformatic sequence analysis

Previously, we identified a partial transcript from our in-house library of putatively immune-related genes (unpublished, Porter) with some similarity to TRAF6/TRAF-like genes. In this study, we confirmed our NGS sequence information using Sanger sequencing for the partial transcript. *Aam*TRAF was screened using the SignalP 6.0 (Teufel et al., 2021), TMHMM 2.0 (Krogh et al., 2001), and conserved domain database (CDD; Marchler-Bauer and Bryant 2004) tools to bioinformatically characterize the sequence. We also screened for sequence similarity between our partial *Aam*TRAF transcript and other publicly available tick sequences. Lastly, we prepared a phylogenetic tree to infer the relationship between compared sequences using the phylogeny.fr tool and the top 20 hits from our BLASTX search. An insect TRAF sequence, from the red mason bee *Osmia bicornis* (XP_029198516.1), was used as the outgroup.

Preparation of AamTRAF dsRNA

Preparation of *Aam*TRAF dsRNA was performed using the *in vivo* L4440/HT115 *E. coli* system. The L4440 plasmid was a gift to Addgene from Andrew Fire for public use (Addgene plasmid #1654; http://n2t.net/addgene:1654; RRID:Addgene_1654). HT115(DE3). Cells were purchased from the Caenorhabditis Genetics Center at the University of Minnesota (https://cgc.umn.edu/strain/HT115(DE3)). To prepare the

expression construct, a 470 bp region of *Aam*TRAF was selected as the dsRNA target region. This region was amplified using the following primers: 5' GGTACCCAAATGTCCCATAGACGGAGAG 3' and 5' AAGCTTCGTGTTCAGGGAACGAGAAA 3'. Restriction enzyme recognition sites, *Kpn*I and *Hin*dIII, respectively, are underlined. To generate amplicons for cloning, the following two-step PCR protocol was used: initial denaturing at 95°C for 3 min followed by 8 cycles of 95°C for 30 sec, 49.9°C for 30 sec, and 72°C for 30 sec, followed by 35 cycles of 95°C for 30 sec, 49.9°C for 30 sec, and 72°C for 30 sec with a final extension step of 72°C for 5 min. Amplicons were subcloned into the L4440 plasmid using standard procedures. Transformation of HT115 cells with the expression construct and expression of dsRNA were performed according to the protocol provided with the HT115 cells. Following dsRNA expression, cells were harvested by centrifugation and resuspended in 500 µl TRIzol reagent (Thermo Fisher, Waltham, MA, USA). A total RNA extraction was performed according to the manufacturer's protocol. Extracted RNA was resuspended in 500 µl DEP-C water and quantified using a spectrophotometer. To confirm the expression of *Aam*TRAF dsRNA, an aliquot of the purified RNA was used in a cDNA synthesis reaction using the Verso cDNA synthesis kit (Thermo Fisher). The presence of *Aam*TRAF dsRNA was subsequently confirmed by standard PCR using the primers previously described.

Tick injections and confirmation of AamTRAF silencing

Ticks used in this study were collected from trails at the McGee State Park in Atoka, OK. Experiments were performed using female ticks. For injections, ticks were immobilized ventral side up on standard laboratory tape under the EZ4W stereomicroscope (Leica Microsystems Inc, Buffalo Grove, IL). A 0.5 µl aliquot (165 ng) of *Aam*TRAF dsRNA was introduced into each tick using a 5 µl syringe fitted with a 33-gauge, 30˚ bevel needle (Hamilton Company, Reno, NV, USA). Injections were placed in the membranous region that occurs between the idiosoma and the fourth coxa. Prior to assessing the effects of silencing, we first determined the dsRNA incubation time required for *Aam*TRAF transcript depletion. For this, four groups of three ticks each were injected with *Aam*TRAF dsRNA and incubated for either 1-, 2-, 4-, or 5- days post-dsRNA introduction. Following the incubation period, total RNA was extracted from each tick individually and cDNA synthesis was performed according to the previously described methods. To confirm silencing of the *Aam*TRAF transcript, the following primers were used, with one primer flanking the dsRNA target region to avoid PCR amplification of the injected *Aam*TRAF dsRNA: 5' CAAGACCAAAGTGTGCAGGC 3' and 5' CGGCTTCGCATCTTGAATGG 3'. Reactions were performed using the T100 Touch (BioRad) according to the following protocol: initial denaturing at 95 ˚C for 3 min, followed by 35 cycles of 95˚C for 30 sec, 51.9˚C for 30 sec, 72˚C for 1 min, and a final extension of 72˚C for 1 min. Primers and conditions for actin PCRs were as previously described (Radulović et al., 2014).

Hemocyte sampling and quantification

The effect of *Aam*TRAF silencing was assessed by quantifying hemocyte proliferation in response to infection with either gram- or gram+ bacteria, *E. coli* and *S. aureus*, respectively. Silencing was performed by injecting ticks with *Aam*TRAF dsRNA as described above, followed by incubation for five days to allow for total transcript silencing, as determined empirically in this study. Following incubation, ticks were infected with either *Escherichia coli* (gram-) or *Staphylococcus aureus* (gram+). To prepare bacteria for infections, overnight cultures of each bacterial species were prepared in LB broth. The following day, bacteria were harvested by centrifugation and washed thrice in diH2O, with final resuspension to an OD_{600} = 0.01. Infections consisted of a 0.5 µl aliquot of prepared bacteria, using the same injection methods as described previously. The infection was allowed to incubate for 24 h before hemolymph collection. A total of 1 µl of hemolymph was retrieved from each tick using a modified Pasteur pipette, with terminal circumference adjusted to fit into the tick tarsus. Collected hemolymph was diluted in 4 µl of 1x PBS and then mixed with trypan blue in a 1:1 ratio, for a total volume of 10 µl. The full 10 µl sample was applied to a cell counting chamber and cells were quantified using the Corning automated cell counter (Corning Inc., Corning, NY, USA) that provides total numbers of live and dead cells, and excludes bacterial cells from counts based on size. The average number of live cells between non-silenced/infected and silenced/infected groups, for each infection type, were compared for statistical significance using a Students T-test assuming equal variance.

RESULTS

Bioinformatic analysis

The *Aam*TRAF sequence was originally identified from our in-house *Amblyomma americanum* transcriptome libraries (unpublished, Porter). The partial transcript represents 728 base pairs, 28 of which are predicted to be in the 5'UTR. A search of the GenBank nucleotide database (discontiguous megablast), using this partial transcript, revealed no sequences with significant similarity. However, screening against protein sequences in GenBank revealed more than 100 hits for sequences in other tick species. Interestingly, none of these hits were for sequences in other *Amblyomma* species. The search also revealed that this partial transcript shares a low identity with other tick transcripts. Percent identities for the top 20 hits ranged between 31.5% and 43% and query coverage values ranged between 38% and 54%. All but three hits were annotated as uncharacterized/hypothetical protein however a predicted domain was detected for all but two of these sequences, and all domain profiles included a RING or MATH domain, or both domains as found in TRAF proteins (Park, 2018). Annotations for the other three hits included RING finger protein 151-like, E3 ubiquitin-protein ligase NRDP1-like, and E3 ubiquitin-protein ligase TRAF7. Due to the similarity of our sequence with TRAF-type sequences, we annotated our contig as *Aam*TRAF. Alignments with other tick TRAF-type sequences showed that our sequence includes the 5' end of the open reading

frame. Domain analysis showed two adjacent C2H2 zinc finger motifs. While a CDD search did not predict a canonical RING domain in this 5' end, a manual sequence screen reveals the following motif: 'C-X2-C-X11-C-X-H-X2-C-X2-C-X10-C-X2,' where only the terminal cysteine is absent as compared to the canonical RING domain. Screening of *Aam*TRAF in TMHMM and SignalP revealed no transmembrane helices and no predicted signal peptide.

Figure 6 shows a phylogenetic tree that demonstrates the relationship between *Aam*TRAF and the closest 20 hits from our GenBank screen. We used a similar TRAF sequence from an insect, the red mason bee, *Osmia bicornis,* as the outgroup (GenBank: XM_029198516.1). Predicted domains for each tick sequence are also indicated in the figure with the exception of KAH7975707.1, which had no predicted domains and no annotation, and for XP_037554938.1 which had no predicted domains and the annotation is shown instead. Sequences clustered into five clades (A-E), and two standalone sequences that include *Aam*TRAF. Aside from *A. americanum*, included sequences were from four tick species, and for each species, TRAF sequences were distributed across multiple clades: A, B, and D for *R. sanguineus,* A and D for *R. microplus,* A, C, and E for *D. silvarum,* and B, D, plus a standalone sequence for *H. asiaticum*. Although *Aam*TRAF is a standalone sequence, node support was low (not shown) and it could be collapsed with clade A. Interestingly, all of the sequences in clade A have either of the standard TRAF RING or the MATH domains but none have both. Additionally, for two sequences, new domains appear to have replaced the RING domain: MOT2 for

KAH7976074.1 and RAD18 for KAH8029893.1. Clade D was also notable in that five of the six sequences contained only the MATH domain. We found no predicted domain in our partial *Aam*TRAF sequence.

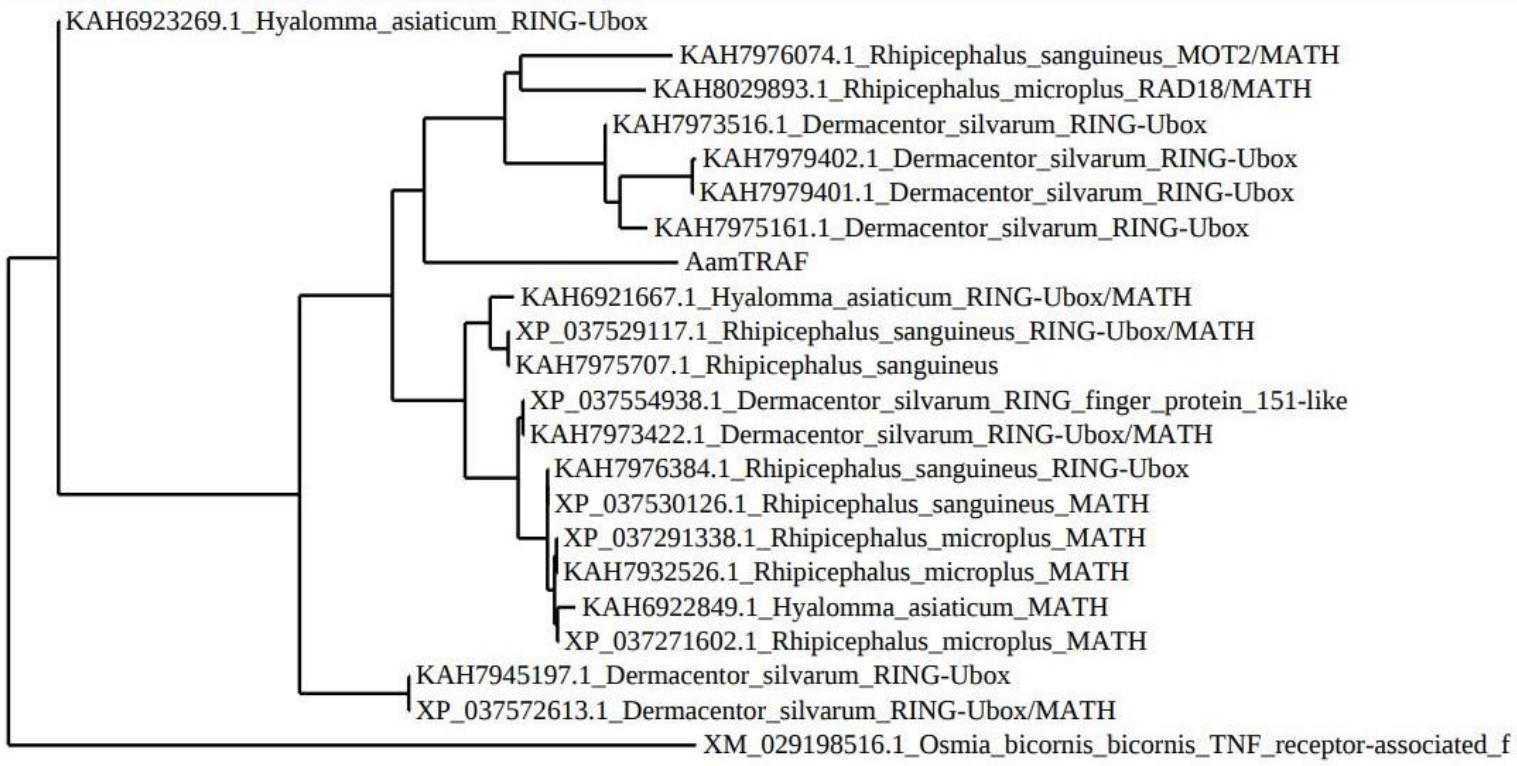

Figure 6. Phylogenetic tree of tick TRAF-like proteins with similarity to *Aam*TRAF**.** The figure shows the phylogentic relationship between *Aam*TRAF with the closest 20 tick sequences in the non-redundant protein database. A TRAF sequence from the red mason bee, *Osmia bicornis*, serves as the outgroup. Tick sequences can be clustered into five clades, A-E, with clade A containing four sequences, clades B-D containing three sequences each, and clade E containing two sequences. Four sequences lack clade association, including *Aam*TRAF. Notably, sequences containing both RING and MATH domains do not fall into a single clade; six of these sequences were distributed into three clades, A, B, and D, while three were not clade-affiliated.

Silencing impact on hemocyte response to bacterial infection

The effects of silencing *Aam*TRAF were analyzed by quantifying the number of hemocytes in infected, non-silenced groups and comparing averages to the respective infected, silenced groups. Average cell counts for live, dead, and total cells for each experimental group are shown in Figure 7. There was no difference in statistical outcome for comparisons using live cells over total cells between experimental groups, therefore total cell counts were used. For gram- infections, out of 15 injected ticks in both non-silenced control and silenced groups, a total of 11 and 12 ticks, respectively, survived the 24 h infection incubation period, respectively. For gram- infected control ticks, averages for live, dead, and total cells were 7.27E+06, 5.87E+05, and 7.86E+06, respectively. For *Aam*TRAF-silenced, gram- infected ticks, averages were 5.69E+06, 3.8E+05, and 6.07E+06, respectively. As seen in Figure 7, comparisons of averages of total cells between these two experimental groups show no statistically significant difference ($p=0.7$) in that hemocyte response to infection with or without silencing of *Aam*TRAF was statistically the same.

For gram+ infections, out of 15 injected ticks in both non-silenced and silenced groups, a total of 15 and 12 ticks, respectively, survived the 24 h infection incubation period. For control ticks, averages for live, dead, and total cells were 5.89E+06, 6.26E+05, and 6.52E+06, respectively. For *Aam*TRAF-silenced ticks, averages were 2.65E+06, 3.03E+05, and 2.95E+06, respectively. Comparisons of averages of total cells between these two experimental groups showed a statistically significant difference

(p=0.009), indicating a significantly higher hemocyte response to infection in control ticks as compared to *Aam*TRAF-silenced ticks. It is also interesting to note that there was no statistical difference between the number of responding hemocytes in gram- infections as compared to gram+ infections ($p = 0.40$), demonstrating that in the absence of *Aam*TRAF silencing, hemocyte proliferation was not statistically different between infection types.

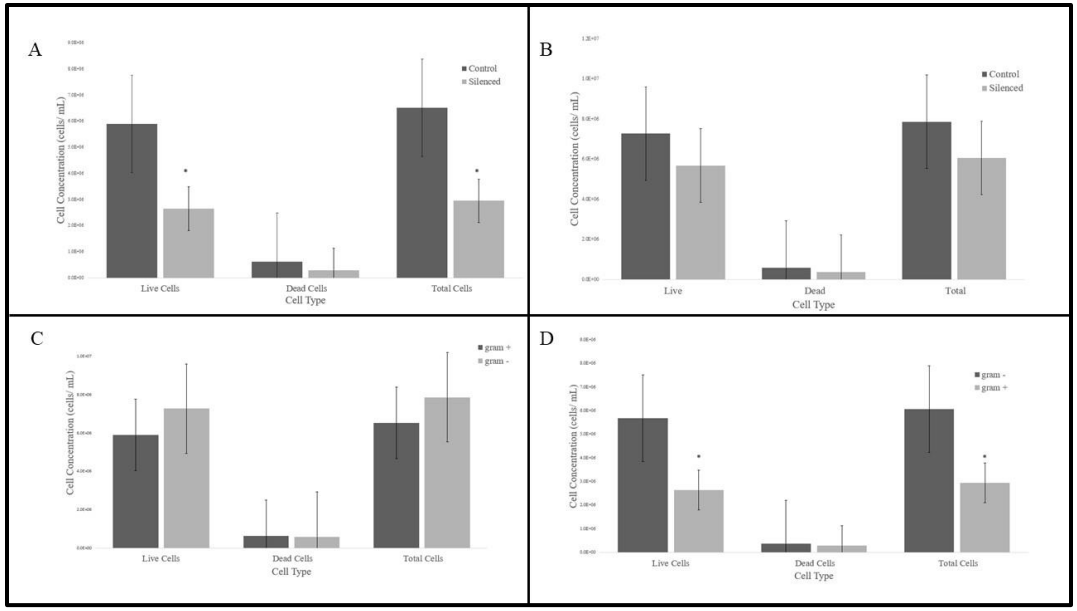

Figure 7. Comparison of hemocyte counts 24 h post-infection in *Aam*TRAF-silenced and control ticks.
The figure shows the average number of live, dead, and total hemocytes for experimental groups. ***Panel A*** shows hemocyte averages for control (non-silenced, darker shaded bars) and *Aam*TRAF-silenced (lighter shaded bars) ticks infected with gram + bacteria (*S. aureus*). Differences in the average number of live and total hemocytes between infection types were statistically significant ($p<0.01$). ***Panel B*** shows hemocyte averages for control (non-silenced, darker shaded bars) and *Aam*TRAF-silenced (lighter shaded bars) ticks infected with gram- bacteria (*E. coli*). Differences in the average number of live, dead, or total hemocyte counts between control and silenced ticks were not statistically significant ($p = 0.69$). ***Panel C*** shows hemocyte averages for gram- (lighter shaded bars) and gram+ (darker shaded bars) infections in ticks, as a comparison of hemocyte production between infection types. Differences in the average number of hemocytes were not statistically significant between infection types ($p = 0.40$). ***Panel D*** shows hemocyte averages for gram- (lighter shaded bars) and gram+ (darker shaded bars) infections in *Aam*TRAF-silenced ticks, as a comparison of infection types in manipulated ticks. Differences in the average number of live and total hemocytes between infection types were statistically significant ($p<0.01$).

DISCUSSION

As ticks are increasingly important vectors of disease-causing pathogens in humans and animals, research into their immune interaction with these microbes has been on the rise. Although a framework of the signaling molecules involved in infection response has been proposed based on insect pathways, putatively involved genes must be validated empirically. In this study, we identified and explored the bacterial infection response involvement of an *Aam*TRAF, putatively functioning in the Toll pathway. The TRAF annotation is based on GenBank screens of our partial transcript that retrieved TRAF-like protein hits. However, identity to retrieved sequences was no higher than ~42%. Additionally, *Aam*TRAF domain analysis reveals that, while mostly conserved, there is a one-cysteine divergence from the canonical RING domain structure. Taken together, these findings suggest that TRAF genes may be evolving relatively fast. This is not surprising given their role in immune response, which can be described as a molecular arms race between the tick host and the microbial pathogen.

Our phylogenetic tree demonstrates that, as with *Aam*TRAF, many tick TRAF-like genes lack the RING/MATH domain profile. Therefore, it is reasonable to hypothesize that more of these unexplored sequences are also involved in the tick immune response. In *I. ricinus* TRAF4, TRAF5-like, and TRAF6-like were shown to interact with viral proteins during infection (Lemasson et al., 2021). Further, previous studies also support a pathogen-specific role for TRAF/TRAF-like proteins. For example,

silencing of HlTRAFD1 in *Haemaphysalis longicornis* decreased tick survival during *E. coli* (gram-) but not *S. aureus* (gram+) infections (Takechi et al., 2015). The authors also found that HlTRAFD1 silencing decreased the expression of the anti-microbial peptide (AMP) *longicin* but not of the gram+-specific H1lysozyme. In *Ixodes scapularis,* a TRAF gene was found to be upregulated in response to tick viral infection, with no change in expression during infection with *Anaplasma phagocytophilum*, a gram- tick-borne bacterium (Mansfield et al., 2017).

Hemocytes mediate much of the immune defense mechanisms that control tick infection (Oliver et al., 2011, Ceraul et al., 2003, Inoue et al., 2001, Buresova et al., 2009, Fiorotti et al., 2019). From this perspective, it is notable that the number of hemocytes present 24 h post-infection was not significantly different between gram+ and gram- infection types. Given that ticks do not vector disease-causing gram+ pathogens, our finding suggests that vector capacity for gram- but not gram+ bacteria is not due to the number of responding hemocytes. Alternatively, certain gram- bacterial species may have evolved mechanisms to overcome hemocyte proliferation pathways in which gram+ bacteria have not. For example, since a lack of *Aam*TRAF prevents hemocyte response, it will be interesting to see if this gene is downregulated during infections with gram- vertebrate pathogens that are transmitted by ticks. Along these lines, our data provide an interesting basis for comparison in that the bacterium used in this study has presumably not evolved to evade or manipulate the tick immune response. Thus, the response here can be compared with the response generated against pathogens that have co-evolved

with these ticks to determine whether they actively suppress immune responses that should normally be present.

The dynamics between gram- and gram+ signaling pathways in ticks are still being resolved, however, there is some evidence that crosstalk between pathways does exist (Olivia Chávez et al., 2017, Fogaça et al., 2021). Given that *Aam*TRAF silencing only affected response to gram+ infection, then if crosstalk does occur, we hypothesize that *Aam*TRAF is not a part of that phenomenon. Instead, our data provide additional support for the division between response pathways for these two bacterial types (Olivia Chávez et al., 2017, Fogaça et al., 2021). Therefore, in light of the data in this and previous studies characterizing functional roles of tick TRAF/TRAF-like proteins, we hypothesize that tick TRAF proteins are pathway-specific and that *Aam*TRAF is a Toll-specific signaling protein.

Emerging evidence points to tick TRAFs as a structurally diverse and multimember group of immune-related molecules that are likely to be pathogen-specific in their role. Of note, we could not find any insect sequences, including in *Drosophila* or mosquitoes, with any significant similarity to *Aam*TRAF. This finding emphasizes the caution required in using insect immune systems to define tick immune systems.

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