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Amblyomma maculatum Immunomodulation in Mammalian Models of Rickettsia parkeri Rickettsiosis

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AMBLYOMMA MACULATUM IMMUNOMODULATION IN MAMMALIAN MODELS OF *RICKETTSIA PARKERI* RICKETTSIOSIS

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ABSTRACT

Rickettsia parkeri is an emerging human pathogen and spotted fever group *Rickettsia* that is transmitted via *Amblyomma maculatum* (the Gulf Coast tick) in the United States. Since these ticks must feed for several days in order to molt to the next life cycle, they must be able to counteract the host immune response. Despite this fact, there have been few studies that evaluate the immunomodulatory effect of this vector and the resultant influence on rickettsial disease. The hypothesis of this research is that, if *A. maculatum* feeding modifies the host immune response, this immunomodulation will enhance disease caused by *R. parkeri*. In order to assess this interaction *in vivo*, rhesus macaques were used to compare intradermal needle inoculation of *R. parkeri* alone to inoculation during *A. maculatum* feeding and *A. maculatum* feeding alone. Tick feeding enhanced local disease and the systemic inflammatory response induced by *R. parkeri*, resulting in increased rickettsial dissemination early in infection, and increased persistence at the inoculation site. In order to quantify the role of *A. maculatum* on the acute rickettsial immune response, C3H/HeN mice were intradermally inoculated with *R. parkeri* both alone and in the presence of *A. maculatum* saliva. The cellular influx of neutrophils and macrophages was significantly downregulated in the *R. parkeri* + saliva group as compared to *R. parkeri* inoculation alone. However, rickettsial load and the cutaneous cytokine response were not significantly modified by *A. maculatum* saliva. Taken together these studies indicate that *A. maculatum* feeding enhances cutaneous pathology in *R. parkeri* rickettsiosis despite the fact that tick saliva inhibits the acute cutaneous cellular infiltrate. Therefore, the immunomodulatory properties of tick feeding cannot be attributed to just the inoculation of saliva alone by the ticks. Future study should evaluate the overall impact of these effects on the establishment of rickettsiosis in the mammalian host in order to develop novel anti-transmission therapeutics.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.1. The Genus *Rickettsia*

The genus *Rickettsia* belongs to the phylum Proteobacteria, the class Alphaproteobacteria, the order Rickettsiales, and the family Rickettsiaceae. Several species of *Rickettsia* have been identified as human pathogens and are transmitted to vertebrate hosts via various hematophagous arthropods (fleas, lice, and mites including ticks) (Table 1.1) (Raoult and Roux 1997, Walker and Ismail 2008). The clinical signs and mortality rate associated with these pathogens is variable, ranging from no reported fatalities (*R. africae, R. parkeri, R. felis, R. akari*) to mortality rates greater than 15% (*R. rickettsii, R. prowazekii*) (Walker and Ismail 2008). Several other species are characterized as endosymbionts of arthropods and have not been definitively identified as human pathogens to date (ex. *R. peacockii, R. rhipicephali, and R. bellii*) (Raoult and Roux 1997, Perlman et al. 2006, Walker and Ismail 2008).

Despite this variable pathogenicity, rickettsial species have several basic characteristics in common. Rickettsiae are small pleomorphic coccobacilli that measure 0.3 to 0.5 µm in width with a variable length from 2 µm to up to 4 µm (Weiss 1973). Atypical "long form" morphology of rickettsiae has been reported for several species including *R. prowazekii*, *R. felis*, and *R. bellii* in both unfavorable and stable environmental conditions where lengths of up to 15 µm have been reported (Gulevskaia et al. 1975, Wisseman et al. 1976, Phillip et al. 1983, Sunyakumthorn et al. 2008). *Rickettsia* are obligate intracellular bacteria that live primarily in the cytoplasm and occasionally the nucleus of host cells (Raoult and Roux 1997). This intracellular niche is required due to the fact that they utilize several host cell molecules such as amino acids, nucleotides, carbohydrates, and enzymes for growth that they cannot synthesize *de novo* (Audia 2012). Furthermore, these organisms are incapable of movement on their own. Therefore, several

Table 1.1 Selected rickettsial diseases in humans (Walker and Ismail 2008).

Disease	Organism	Arthropod vector	Eschar	Rash	Regional lymph-adenopathy	Symptoms or fever	Mortality rate*
Tick-transmitted spotted fevers							
Rocky Mountain spotted fever	*Rickettsia rickettsii*	*Dermacentor variabilis, Dermacentor andersoni, Rhipicephalus sanguineus, Amblyomma cajennense,* and *Amblyomma aureolatum*	Rare	Yes	No	Yes	High
Boutonneuse fever	*Rickettsia conorii*	*Rh. sanguineus, Rhipicephalus pumilio*	Frequent	Maculopapular	No	Yes	Mild to moderate
African tick bite fever	*Rickettsia africae*	*Amblyomma hebraeum, Amblyomma variegatum*	Frequent and often multiple	Papular or vesicular; often sparse or absent	Yes	Yes	None reported
Maculatum disease	*Rickettsia parkeri*	*Amblyomma maculatum, Amblyomma triste*	Yes	Often	Yes	Yes	None reported
Flea-transmitted diseases							
Flea-borne spotted fever	*Rickettsia felis*	*Ctenocephalides felis*	Sometimes	Sometimes	No	Yes	None reported
Murine typhus	*Rickettsia typhi*	*Xenopsylla cheopis, Ctenocephalides felis*	No	Yes	No	Yes	Low
Louse-transmitted disease							
Epidemic typhus	*Rickettsia prowazekii*	*Pediculus humanus humanus*	No	Yes	No	Yes	High
Epidemic typhus	*Rickettsia prowazekii*	Fleas and lice of flying squirrels (*Glaucomys volans volans*)	No	Yes	No	Yes	Low
Mite-transmitted diseases							
Rickettsialpox	*Rickettsia akari*	*Liponyssoides sanguineus*	Yes	Yes	Yes	Yes	None reported

*High mortality is >15%; moderate mortality is 7–15%; mild-to-moderate mortality is 2–7% and low mortality is ≤1%.

species have the ability to polymerize host cell actin, forming actin tails for intracellular movement (Teysseire et al. 1992, Heinzen et al. 1993, Simser et al. 2002, Ogata et al. 2005, Ogata et al. 2006, Serio et al. 2010). Future investigations are needed to determine how this cell-assisted motility impacts rickettsial pathogenicity.

Rickettsiae have fine ultrastructural morphology similar to other Gram negative bacteria, e.g. *Escherichia coli* (Figure 1.1). They possess a cell envelope that contains an inner membrane surrounded by a thin layer of peptidoglycan and an outer membrane that contains lipopolysaccharide (LPS) and is surrounded by a capsule (Anacker et al. 1967, Anacker et al. 1984, Teysseire and Raoult 1992). LPS elicits a strong immune response via activation of host Toll-like receptor 4 (TLR4). This TLR4-mediated response is important in the rickettsial immune response, as demonstrated by C3H/HeJ mice with defective TLR4 that have increased susceptibility to rickettsial infections (Jordan et al. 2008, Grasperge et al. 2012). Rickettsiae also possess proteins that insert into the outer membrane of the cell envelope that are called surface cell antigens (Sca). Some of these proteins have been shown to play an important role in rickettsial-host cell interactions. For example, rickettsial outer membrane protein B (rOmpB) binds Ku70 on mammalian cells for adhesion and subsequent invasion (Chan et al. 2009). Other proteins, such as rOmpA, Sca2, and Sca1 also play a role in *Rickettsia*-host cell binding and invasion, although their receptors on host cells have yet to be identified (Li and Walker 1998, Cardwell and Martinez 2009, Chan et al. 2010, Riley et al. 2010).

1.1.1. Rickettsial Classification

The genus *Rickettsia* has recently been reclassified into four groups based on phylogenetic analysis: spotted fever group (SFG), typhus group, transitional group, and ancestral group (Table 1.2) (Gillespie et al. 2007, Sahni et al. 2013). Species within the SFG are associated

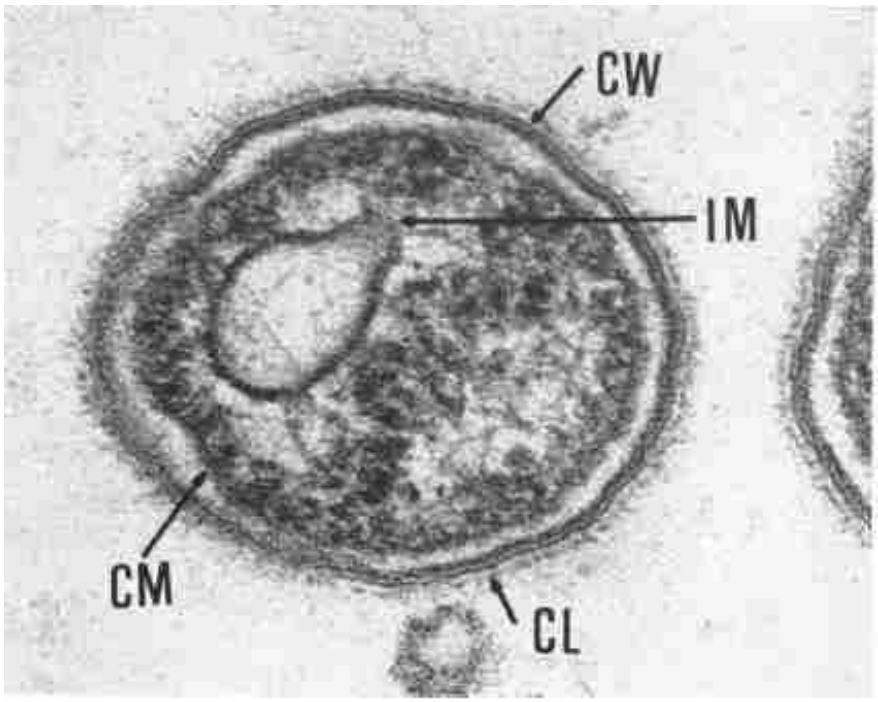

Figure 1.1. The fine structure of *R. prowazekii* as revealed in the thin section of the chick yolk sac. CL = capsule-like structure, CM = cytoplasmic membrane, CW = cell wall, IM = intracytoplasmic membrane (Anacker et al. 1967).

Table 1.2. Select *Rickettsia* species divided into subgroups (adapted from (Sahni et al. 2013)).

Spotted fever group	Typhus group
R. africae	*R. prowazekii*
R. conorii	*R. typhi*
R. heilongjiangensis	
R. helvetica	Transitional group
R. honei	*R. australis*
R. japonica	*R. akari*
R. massiliae	*R. felis*
R. montanensis	
R. parkeri	Ancestral group
R. peacockii	*R. bellii*
R. philipi	*R. canadensis*
R. rhipicephali	
R. rickettsii	
R. sibirica	
R. slovaca	
R. endosymbiont of *Ixodes scapularis*	
R. amblyommii	

with hard ticks belonging to the Ixodidae family. This rickettsial group contains species that have been identified as human pathogens as well as species that have no known human pathogenicity. The characteristics of SFG *Rickettsia* will be discussed further in the next section. The typhus group contains two human pathogens, *R. prowazekii* and *R. typhi* that are transmitted by lice and fleas, respectively (Gillespie et al. 2007, Sahni et al. 2013). The name typhus arises from the Greek word typhos meaning smoky or hazy referring to the predilection for central nervous system involvement and the altered mental state frequently associated with these pathogens (Dumler 2012). The ancestral group consists of two species, *R. bellii* and *R. canadensis* that have no known pathogenicity and appear to be restricted to their tick hosts. The transitional group contains three human pathogens, *R. australis*, *R. akari*, and *R. felis* transmitted by ticks, mites, and fleas, respectively. *R. felis* possesses characteristics that are common to both the typhus and SFG of *Rickettsia* (Gillespie et al. 2007). Similar to members of the typhus group, *R. felis* is transmitted by insects and has hemolytic activity (Gillespie et al. 2007). However, *R. felis* is serologically cross-reactive to SFG *Rickettsia*, can polymerize actin, and is maintained transovarially within its vector like the SFG species (Gillespie et al. 2007). Interestingly, plasmids have been found within the ancestral and transitional groups and members of the SFG with no known pathogenicity, but not within highly pathogenic species of *Rickettsia*, such as *R. rickettsii*, *R. prowazekii*, or *R .conorii* (Gillespie et al. 2007, Baldridge et al. 2010). This suggests that primitive *Rickettsia* species contained plasmids, but they were lost as these species gained pathogenicity due to the fitness cost associated with retaining plasmids (Gillespie et al. 2007). The remainder of this section will focus on SFG *Rickettsia*, particularly the pathogen of interest for the subsequently presented studies, *R. parkeri*.

1.1.2. Spotted Fever Group *Rickettsia* Pathogenesis

The members of the SFG of *Rickettsia* are currently classified together based on phylogenetic and bioinformatic analysis of rickettsial genomes (Gillespie et al. 2007). Despite the fact that this group contains both human pathogens and species of no known pathogenicity, there are several general characteristics that these bacteria share. These bacteria express the surface protein rOmpA, which is absent in typhus group *Rickettsia* (Blanc et al. 2005). The role of rOmpA in rickettsial infection has not been fully elucidated. In murine fibroblast cells, antibodies against rOmpA inhibited rickettsial adherence (Li and Walker 1998). However, recently it was shown that an *R. rickettsii* rOmpA knock out did not result in attenuation of virulence when compared to the wildtype infection (Noriea et al. 2015). SFG *Rickettsia* are also all maintained transovarially and transstadially in tick vectors as will be described in the next section (Gillespie et al. 2007, Macaluso and Paddock 2013). Lastly, most SFG *Rickettsia* harness host cell actin for intracellular movement in a similar fashion. With the exception of *R. peacockii*, both pathogenic and non-pathogenic members of the SFG of *Rickettsia* have been shown to consistently polymerize host actin, forming long straight actin tails (Heinzen et al. 1993, Simser et al. 2002, Ogata et al. 2005, Serio et al. 2010). This is in contrast to typhus group organisms that either do not associate with actin – *R. prowazekii*, or form short hooked tails – *R. typhi* (Figure 1.2) (Teysseire et al. 1992, Heinzen et al. 1993).

1.1.2.a. Maintenance and Transmission by Tick Vectors

As previously mentioned, SFG *Rickettsia* are found within and transmitted to vertebrate hosts including humans by hard tick vectors. These bacteria are primarily maintained in the wild tick populations via transovarial and transstadial transmission (Figure 1.3) (Eremeeva and Dasch 2015). These routes of transmission are essential for rickettsial maintenance as they allow for

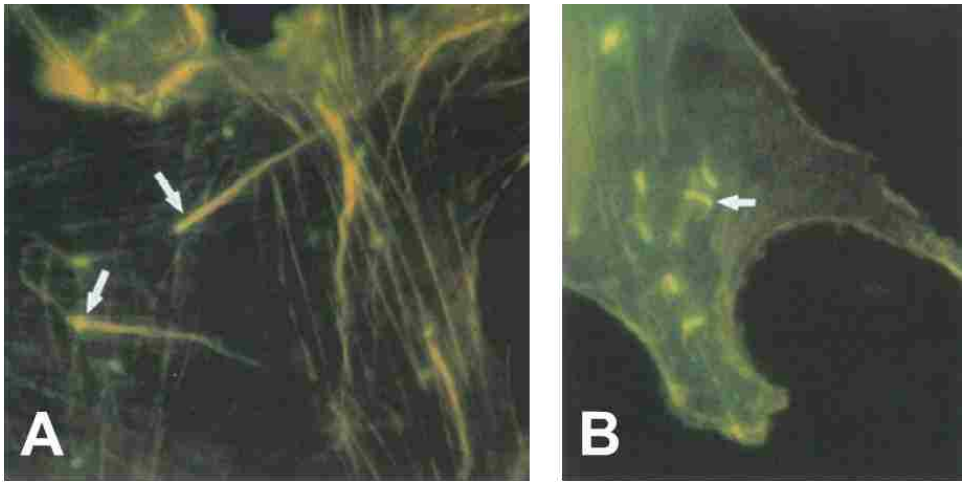

Figure 1.2 Dual fluorescent staining of rickettsiae and F-actin in infected Vero cells. (A) Doubly stained Vero cells infected for 23 h with virulent *R. rickettsii* R strain showing fluorescein-labeled *R. rickettsii* R (apple green) and F-actin (yellow). Note the colocalization of F-actin fibrils with one pole of *Rickettsia*, giving the appearance of a tail. (F) *R. typhi* with short, hook-shaped F-actin tails. Arrows identify typical organisms. All panels are of equal magnification (adapted from (Heinzen et al. 1993)).

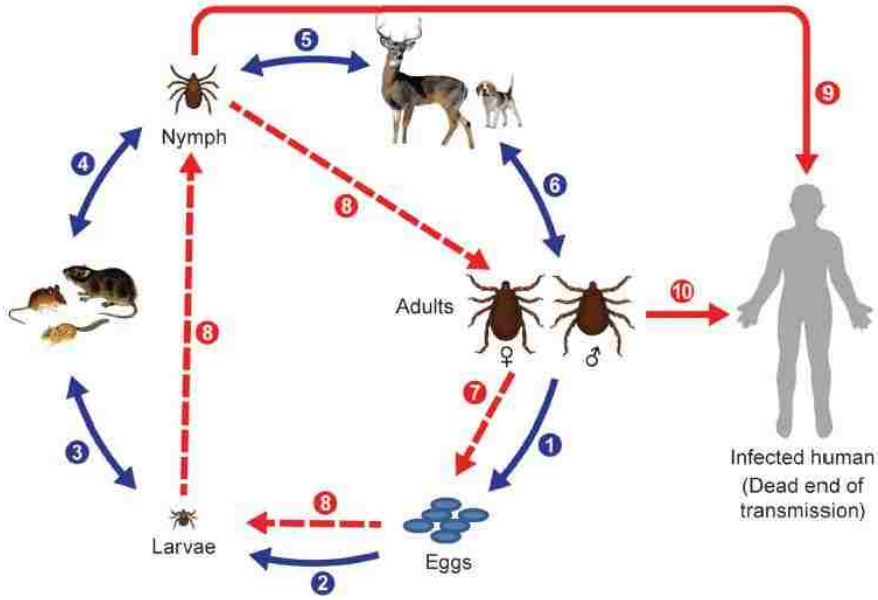

Figure 1.3. Life cycle of Ixodid ticks and natural transmission of rickettsiae. Blue arrows indicate main steps of the tick natural cycle: (1) oviposition by engorged female; (2) eggs hatched into larvae; (3) larvae feed on small animals; (4) engorged larvae hatch into nymphs; (5) nymphs feed on large or small animals; and (6) nymphs molt into adult ticks that feed on large animals or bite humans. Broken red arrows indicate transovarial (7) and transstadial transmission (8) of rickettsiae, and solid red arrows indicate transmission of rickettsiae to humans through a bite of a nymph (9) or an adult tick (10) (Eremeeva and Dasch 2015).

persistence of rickettsial infection in the environment without the need for an infected vertebrate host, and can result in significant expansion of the numbers of infected ticks given that a single engorged female can potentially produce thousands of infected eggs (Eremeeva and Dasch 2015). Furthermore, since hard ticks molt to a new life stage after each feeding, transstadial transmission is essential to ensure that newly molted ticks will be infectious to their next vertebrate host (Socolovschi et al. 2009c). That being said, the success of these transmission routes varies depending on the species and strain of *Rickettsia* (Socolovschi et al. 2009c, Eremeeva and Dasch 2015). The rates of successful transovarial transmission for rickettsial endosymbionts, such as *R. peacockii* and *R. amblyommii* have been shown to be 100% in their respective tick hosts (Simser et al. 2001, Stromdahl et al. 2008). In contrast, the transovarial transmission rates for strains of the pathogen *R. rickettsii* vary from 35-100% in *Dermacentor andersoni* ticks (Price 1954, Burgdorfer and Brinton 1975). The reasons for this variability in transmission success are unknown, but may be associated with a fitness cost to the tick host as a result of rickettsial infection. Infections with *R. peacockii* and *R. montanensis* (another endosymbiont) have no detrimental effect on their respective tick hosts (Niebylski et al. 1999, Macaluso et al. 2002). However, infections of *D. andersoni* with the virulent strains of *R. rickettsii* have been shown to have a detrimental effect on tick survival, molting success, oviposition, and fecundity (Niebylski et al. 1999). For other rickettsial species such as *R. conorii*, there is a strain-dependent fitness cost to rickettsial infection, where infections with certain strains do not affect the tick host and others adversely affect molting success and survival (Matsumoto et al. 2005, Levin et al. 2009, Socolovschi et al. 2009a, Socolovschi et al. 2009b).

In tick-*Rickettsia* pairs with a low success rate of transovarial transmission, infected vertebrate hosts may be a source for ticks to acquire rickettsial infection. In this scenario, human

beings are thought to be dead end incidental hosts of the bacteria when fed upon by infected ticks and do not participate in further transmission of the pathogen (Figure 1.3) (Eremeeva and Dasch 2015). Several wild and domestic animals such as mice, rats, quail, squirrels, chipmunks, rabbits, hares, woodchucks, opossums, deer, black bears, dogs, cats, and cattle have been shown to be rickettsemic, seropositive, and/or harbor *Rickettsia*-infected ticks in natural and experimental settings (reviewed in (Eremeeva and Dasch 2015)). Ricketts was able to infect squirrels, groundhogs, chipmunks, and rats with blood from Rocky Mountain spotted fever (RMSF) patients and ticks fed on guinea pigs that were inoculated with blood from RMSF patients (Ricketts 1909). Blood from these animals was then subsequently used to produce disease in naïve guinea pigs (Ricketts 1909). Several decades later, Burgdorfer demonstrated that meadow mice, squirrels, hares, and chipmunks developed rickettsemia in response to intraperitoneal inoculations of *R. rickettsii* and ticks fed on these animals during peak rickettsemia acquired the infection (Burgdorfer et al. 1966). While these experiments demonstrate that rickettsemic animals can serve as potential sources for rickettsial infection of ticks, the extent to which these infections can be transmitted to humans has not been determined.

Ticks can also transmit *Rickettsia* to each other via co-feeding, where an uninfected tick acquires rickettsial infection from an infected tick feeding in close proximity on the same animal host. This route of transmission does not require rickettsemia within the host, and has been experimentally demonstrated for at least two SFG *Rickettsia* species (Zemtsova et al. 2010, Wright et al. 2015b). In the first study, experimentally-infected adult *Rhipicephalus sanguineus* transmitted *R. conorii* to uninfected nymphs that co-fed at the same location on dogs (Zemtsova et al. 2010). In the second study, uninfected lone star tick (*Amblyomma americanum*) nymphs acquired *R. parkeri* infection after feeding with naturally-infected, adult *A. maculatum* on guinea

pigs (Wright et al. 2015b). In both cases, the infection was maintained transstadially after one molt (Zemtsova et al. 2010, Wright et al. 2015b). Lastly, sexual transfer of SFG *Rickettsia* between infected males and uninfected females has been described in *Ixodes ricinus* ticks; however, subsequent transovarial transmission was unsuccessful, indicating that this transmission route may not contribute to rickettsial maintenance in nature (Hayes et al. 1980).

1.1.2.b. Established and Emerging SFG Rickettsioses in the United States

RMSF caused by *R. rickettsii* has historically been recognized as the most prevalent and most pathogenic SFG rickettsial disease in the United States. This disease was first described in 1896 as a spotted fever of an unknown origin in an annual report of the Surgeon General (Spencer 1929). In 1906, Ricketts was the first to establish an animal model of RMSF in guinea pigs and monkeys (Ricketts 1906a). He was also the first to implicate the tick as a vector for this pathogen, demonstrating that ticks fed on infected guinea pigs could be used to transmit the disease-causing agent to other guinea pigs as well as other small animals (Ricketts 1906b, Ricketts 1909). The most consistent clinical signs of RMSF include headache, rash, and fever with the fever usually developing first and the rash as the hallmark of the disease occurring several days after the fever (Kirk et al. 1990, Cunha 2008, Lin and Decker 2012). Severe sequelae of RMSF include hypovolemia, hypotension, and rarely disseminated intravascular coagulation, which occur as a result of vascular injury due to multiplication of bacteria within vascular endothelium (Harrell and Aikawa 1949, Rao et al. 1988, Elghetany and Walker 1999, Lin and Decker 2012). Complications that can occur in severe RMSF include central nervous system involvement due to meningitis or meningoencephalitis, renal, hepatic, and pulmonary dysfunction (Lin and Decker 2012). Death can occur in 7 to 15 days after onset of clinical symptoms if appropriate antibiotic therapy is not instituted early in the course of disease (Lin and

Decker 2012). Doxycycline is the treatment of choice for RMSF and should be administered as soon as possible based on clinical suspicion to avoid serious sequelae (Lin and Decker 2012).

In the 2000's, at least two other SFG rickettsioses have emerged as human pathogens: *R. parkeri* rickettsiosis and *Rickettsia* 364D rickettsiosis in the United States. These rickettsioses are just two examples of a number of emerging rickettsioses diagnosed around the world that were discovered several to many years after the original description of the rickettsial species itself (Table 1.3) (Paddock 2009). It is also important to note that these emerging pathogens were originally misdiagnosed as other established rickettsioses likely due to the overlap in clinical signs between these rickettsioses and cross-reaction on commonly used diagnostic tests (Paddock 2009). While the exact reasons for the emergence of these pathogens is unclear, other rickettsial species have been identified as having the potential for emergence as pathogens based on the fact that they are found in ticks that may bite humans and that they have been demonstrated to cause disease in animals (Table 1.4) (Paddock 2009).

In 2004, the first case of *R. parkeri* rickettsiosis was reported in a man in Southeast Virginia (Paddock et al. 2004). This was the first report of SFG rickettsiosis not attributed to *R. rickettsii* in the United States. Through 2014, at least 37 cases of *R. parkeri* rickettsiosis have been identified (Whitman et al. 2007, Paddock et al. 2008, Cragun et al. 2010, Myers et al. 2013, Ekenna et al. 2014, Kaskas et al. 2014, Paddock and Goddard 2015). Clinically, *R. parkeri* rickettsiosis (also called "Tidewater spotted fever", "American boutonneuse fever", and "Maculatum rickettsiosis") is defined by a fever, headache, maculopapular rash, myalgia, and the presence of multiple eschars (Paddock et al. 2004, Whitman et al. 2007, Paddock et al. 2008, Cragun et al. 2010, Jiang et al. 2012, Ekenna et al. 2014, Kaskas et al. 2014). An eschar is defined as a 0.5–2 cm in diameter, crusted, non-pruritic ulcer, surrounded by an indurated,

Table 1.3. Interval between discovery of selected rickettsiae and confirmation of these agents as pathogens of humans (Paddock 2009).

Agent	Year of discovery (initial designation)	Year reported as a confirmed pathogen (interval from discovery)	Initial diagnosis of index patient(s)
Rickettsia parkeri	1937 (maculatum agent)	2004 (67)	Rickettsialpox
R. honei	1962 (TT-118)	1992 (30)	Queensland tick typhus
R. slovaca	1968 (strains B, D)	1997 (29)	Lyme borreliosis
R. felis	1990 (ELB agent)	1994 (4)	Murine typhus
R. massiliae	1992 (strains Mtu1, Mtu5)	2006 (14)	Mediterranean spotted fever (MSF)
R. aeschlimannii	1995 (strain PoTiR8)	2002 (7)	MSF
R. raoultii	1999 (genotypes RpA4, DnS14, DnS28)	2006 (7)	Tick-borne lymphadenopathy
R. monacensis	2002 *(R. monacensis*)	2007 (5)	MSF
Rickettsia 364D	1966 (serotype 364D)	2010 (44)	RMSF, anthrax

Table 1.4. Characteristics of selected North American rickettsiae of suspected or undetermined pathogenicity in humans (adapted from (Paddock 2009)).

Rickettsia species or strain	Tick(s) infected with *Rickettsia* in nature	Frequency with which tick(s) will bite humans	Demonstrated pathogenicity of *Rickettsia* (route of infection)
Rickettsia bellii	Multiple genera, including *Dermacentor* and *Amblyomma*	Frequent	Eschars in rabbits and guinea pigs (ID)
Rickettsia canadensis	Multiple genera, including *Haemaphysalis* and *Dermacentor*	Infrequent to Frequent	Fever in guinea pigs (IP)
Strain Parumapertus	*Dermacentor parumapertus*	Infrequent	Fever and scrotal erythema in guinea pigs (IP)
Strain Tillamook	*Ixodes pacificus*	Frequent	Death in mice (IP)
Rickettsia rhipicephali	Multiple genera, including *Dermacentor* and *Rhipicephalus*	Frequent	Fever, scrotal swelling, and death in meadow voles (IP)

ID = intradermal, IP = intraperitoneal

erythematous halo (Figure 1.4) (Paddock et al. 2004). Histologically, extensive necrosis of the epidermis and superficial dermis and prominent lymphohistiocytic vasculitis of dermal vessels is present (Figure 1.5) (Paddock et al. 2004, Paddock et al. 2008, Cragun et al. 2010, Kaskas et al. 2014). Clinically, *R parkeri* rickettsiosis can be differentiated from RMSF by the presence of an inoculation eschar and milder illness (Table 1.5) (Paddock et al. 2004, Paddock et al. 2008, Paddock and Goddard 2015). Less than a third of the patients diagnosed with *R. parkeri* rickettsiosis require hospitalization, and to date there have been no reported central nervous system manifestations or fatalities (Paddock and Goddard 2015). Similar to RMSF, patients respond favorably to treatment with doxycycline (Paddock et al. 2004, Paddock et al. 2008).

In addition to *R. parkeri*, *Rickettsia* 364D, another SFG *Rickettsia*, was recently discovered as a human pathogen in the United States with the first case series of four individuals reported in California in 2010 (Shapiro et al. 2010). Subsequently, three more cases were diagnosed in children in California (Johnston et al. 2013). All of these patients presented with an eschar with only one child developing a macular rash (Shapiro et al. 2010, Johnston et al. 2013). More than half of the patients also presented with fever, fatigue, headache, lymphadenopathy, and myalgia/arthralgia (Shapiro et al. 2010, Johnston et al. 2013). All of the patients responded to treatment with doxycycline without the need for hospitalization (Shapiro et al. 2010, Johnston et al. 2013). Due to the fact that *R. parkeri* and *Rickettsia* 364D were recently described as pathogens, there is a lack of knowledge and need for research regarding their biology and how their vectors influence the pathogenesis of these organisms. Additionally, these rickettsiae may have an impact on the epidemiology of SFG rickettsioses in the United States as will be discussed in the next section.

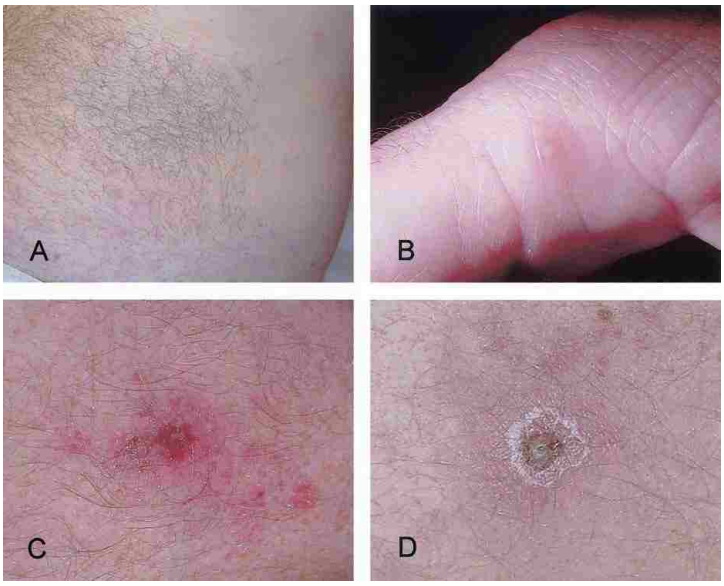

Figure 1.4. Cutaneous lesions in a patient infected with *R. parkeri*. A. A diffuse, pink macular rash involving the abdomen. B, A small pustule on the medial aspect of the first digit. C and D, Eschars located on the pretibial aspects of the right and left lower legs, respectively (Paddock et al. 2004).

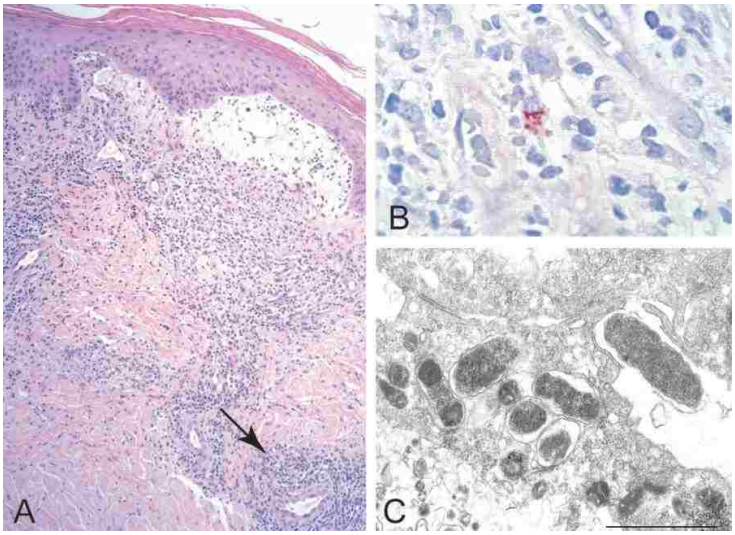

Figure 1.5. Histopathologic and immunohistochemical evaluation of a biopsy specimen from the margin of an eschar, and ultrastructure of *R. parkeri* (strain Portsmouth) isolated in cell culture. A, Lymphohistiocytic perivascular infiltrates (*arrow*, representative focus) involving the superficial and deep dermis, and subepidermal blistering at the periphery of the eschar represented grossly in figure 1.4D (hematoxylin and eosin stain; original magnification x25). B, Immunohistochemical staining of SFG rickettsiae *(red)* in the cytoplasm of a cell in a focus of perivascular inflammation (immunoalkaline phosphatase with naphthol-fast red substrate and hematoxylin counterstain; original magnification x250). C, Ovoid and rod-shaped bacteria in the cytoplasm of a Vero E6 cell (an electron micrograph; uranyl acetate and lead citrate stain; bar equals 1 mm) (Paddock et al. 2004).

Table 1.5. Comparison of the clinical characteristics of *R. parkeri* rickettsiosis and Rocky Mountain spotted fever (RMSF) in the United States (Paddock and Goddard 2015).

Characteristic	*R. parkeri* Rickettsiosis (n = 21)	RMSF (n = 398)
	Percentage of patients with characteristic[a]	
Fever	100	99
Inoculation eschar	95	NR[b]
Any rash	90	92
Any macules or papules	86	53
Any vesicles or pustules	33	NR[b]
Any petechiae	14	52
Headache	86	80
Myalgia	76	60
Nausea or vomiting	10	66
Diarrhea	0	25
Coma, seizures, delirium, or confusion	0	27
Death	0	8

[a]Percentages determined from the number of patients for whom the clinical characteristic was specifically evaluated. [b]NR, not reported in the clinical description of any case series.

1.1.2.c. The Evolving Eco-epidemiology of SFG *Rickettsia* and Their Tick Vectors in the United States

From 2000-2013, there has been a 6-fold increase in the number of human cases of SFG *Rickettsia* per 100,000 people that were reported to the Centers for Disease Control and Prevention (CDC) in the United States (Figure 1.6) (Koo et al. 1994, Groseclose et al. 2004, Adams et al. 2015). The exact cause of this dramatic increase in incidence is unknown, but it is likely multifactorial due to a combination of a true increase in disease as well an increase in incidence rate due to increased reporting. One of the potential causes for true increased incidence is the emergence of previously unrecognized SFG rickettsioses that clinically resemble RMSF. In fact, approximately 95% of the cases of RMSF between 1981 and 2005 were diagnosed via serologic tests that do not differentiate between SFG rickettsial species (Paddock et al. 2008). Therefore it is possible that other SFG *Rickettsia*, other than *R. rickettsii*, have contributed to the

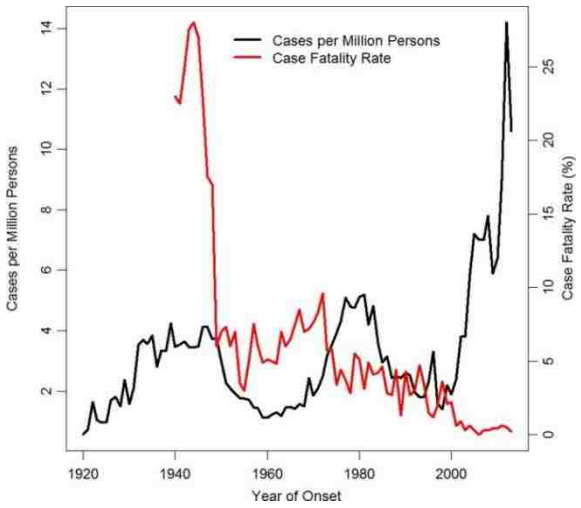

Figure 1.6. Historical incidence rate and case fatality rate of spotted fever group rickettsiosis in the United States, 1920–2013. Depicted is a dramatic rise in disease incidence over the last 15 years with a concurrent decrease in case fatality rate (Koo et al. 1994, Groseclose et al. 2004, Openshaw et al. 2010, Adams et al. 2015, Dahlgren et al. 2016, Drexler et al. 2016)

overall increase in SFG rickettsiosis. Thus, in 2009, the terminology for reporting rickettsial diseases to the CDC in the United States was changed from cases of RMSF to SFG rickettsioses to include other SFG pathogens such as *R. parkeri*. Additionally, as the incidence of SFG *Rickettsia* has increased, there has been a concomitant decrease in fatality rate (Figure 1.6) (Dahlgren et al. 2016). This decreased fatality rate implicates that these cases of SFG rickettsioses may be due to less pathogenic emergent SFG rickettsiae, like *R. parkeri,* instead of *R. rickettsii* (Raoult and Parola 2008). In fact, one report indicated that one-third of cases diagnosed as RMSF by immunofluorescence antibody tests were actually caused by *R. parkeri* based on western blot (Raoult and Paddock 2005). *R. amblyommii*, a SFG species of unknown pathogenicity, carried by *A. americanum* has also been suggested to play a role in the changing epidemiology of SFG rickettsiosis in the United States (Apperson et al. 2008, Dahlgren et al. 2016). This proposed role is based on the high prevalence of *R. amblyommii* in lone star ticks, the overlap in tick range with the majority of SFG *Rickettsia* cases, serologic cross-reactivity to *R. rickettsii*, and little to no pathogenicity demonstrated for the organism (Dahlgren et al. 2016).

While the increase in SFG rickettsial cases can partially be explained by the emergence of newly reported rickettsial pathogens, a new tick vector for *R. rickettsii* has also recently been identified. The only previously recognized vectors of *R. rickettsii* in the United States are *D. andersoni*, the Rocky Mountain wood tick, found in the Western United States (Figure 1.7), and *D. variabilis*, the American dog tick, in the Eastern and Central United States (Figure 1.8). However, a newly recognized vector, *Rh. sanguineus*, (the brown dog tick) was responsible for transmission of *R. rickettsii* during an outbreak of RMSF in Arizona in 2002 (Demma et al. 2005). The presence of *R. rickettsii* in these ticks may have an impact on RMSF incidence as *Rh. sanguineus* are found across the entire United States (Figure 1.9). Lastly, while there likely is a true increase in incidence of SFG *Rickettsia*, there has also been an increase in reported incidence due to an increase in surveillance. This increased surveillance can be attributed to newly implemented, standardized, national guidelines for reporting of SFG rickettsial disease as well as increased federal funding of public health programs (Openshaw et al. 2010).

Figure 1.7. Approximate distribution of the Rocky Mountain Wood tick, *D. andersoni* in the United States. (Courtesy of Centers of Disease Control and Prevention)

Figure 1.8. Approximate distribution of the American dog tick, *D. variabilis* in the United States. (Courtesy of Centers of Disease Control and Prevention)

Figure 1.9. Approximate distribution of the brown dog tick, *Rh. sanguineus* in the United States. (Courtesy of Centers of Disease Control and Prevention)

1.1.2.d. *Rickettsia parkeri* Ecology

The primary vector of *R. parkeri* is believed to be the Gulf Coast tick, *Amblyomma maculatum*. This is supported by the fact that *R. parkeri* is transovarially maintained in *A. maculatum* and has been found in ovaries and salivary glands in infected ticks (Edwards et al. 2011, Wright et al. 2015a). Furthermore, all of the cases of *R. parkeri* rickettsiosis have been

reported in the thirteen states where *R. parkeri* was also detected in *A. maculatum* (Figure 1.10) (Paddock and Goddard 2015). The percentage of *R. parkeri* positive *A. maculatum* found either free in the environment or attached to vertebrate hosts in these states varies from 1.4% to 65% with the highest prevalence reported from surveys in Virginia, Louisiana, and Mississippi (Sumner et al. 2007, Cohen et al. 2009, Paddock et al. 2010, Trout et al. 2010, Fornadel et al. 2011, Varela-Stokes et al. 2011, Wright et al. 2011, Ferrari et al. 2012, Jiang et al. 2012, Florin et al. 2013, Leydet and Liang 2013, Florin et al. 2014, Nadolny et al. 2014, Pagac et al. 2014, Mays et al. 2016). Interestingly, in several field surveys from Oklahoma and Kansas including hundreds of ticks, only one *R. parkeri* positive *A. maculatum* has been found in Oklahoma and none in Kansas (Sumner et al. 2007, Jiang et al. 2012, Barrett et al. 2014, Paddock et al. 2015). Concurrently, surveys have found high prevalence of the endosymbiont *Candidatus* "R. andeanae" in these tick populations, suggesting that this symbiont may interfere with *R. parkeri* acquisition in these ticks (Paddock et al. 2015). However, as no such interference effect has been experimentally confirmed for *Candidatus* "R. andeanae", further research is needed. In South America, *Amblyomma triste* and *Amblyomma tigrinum* are implicated as the primary vectors of *R. parkeri* rickettsiosis based on finding these infected tick species in regions where *R. parkeri* rickettsiosis has been diagnosed (Romer et al. 2011, Portillo et al. 2013, Romer et al. 2014).

R. parkeri DNA has also been detected in a variety of other hard tick species including *A. americanum*, *D. variabilis*, *Rh. sanguineus*, *Haemaphysalis leporispalustris*, and *Ixodes scapularis* in the United States and *Amblyomma dubitatum* and *Amblyomma nodosum* in South America (Cohen et al. 2009, Ogrzewalska et al. 2009, Leydet and Liang 2013, Gaines et al. 2014, Henning et al. 2014, Lado et al. 2014). There is evidence that *A. americanum* may be able to acquire and transmit *R. parkeri*, as experimentally infected ticks have been shown to maintain

Figure 1.10. Case-patient locations of *R. parkeri* rickettsiosis in the United States, 2002–2014. States where *R. parkeri* has been identified in adult Gulf Coast ticks are shaded gray (Paddock and Goddard 2015).

this infection transstadially and transovarially (Goddard 2003). Furthermore, guinea pigs exposed to these ticks developed a fever and scrotal reactions (Goddard 2003). However, sequencing of rickettsial amplicons was not performed in infected ticks casting some doubt on the validity of these results. *A. americanum* have also been shown to acquire *R. parkeri* while feeding concurrently with infected *A. maculatum* on guinea pigs and then transstadially maintain this infection, indicating the possibility of spread of *R. parkeri* between tick populations (Wright et al. 2015b). On the other hand, infestation by *A. americanum* has not been reported in documented cases of *R. parkeri* rickettsiosis and field surveys have shown that ≤ 1% wild-caught *A. americanum* are *R. parkeri* positive (Cohen et al. 2009, Gaines et al. 2014). Therefore, despite their large, expanding range, abundant numbers in nature and predilection for biting humans (Paddock and Yabsley 2007, Dahlgren et al. 2016), the role of these ticks in the transmission of *R. parkeri* rickettsiosis is unknown.

1.1.2.e. SFG *Rickettsia* of Unknown Pathogenicity

In addition to the known pathogens that belong to the SFG of *Rickettsia*, there are several species that have been classified as symbionts of their associated tick species and have yet to be definitively identified as human pathogens. However, it is still important to study these organisms as they affect pathogen ecology and may emerge as pathogens in the future. Some examples of SFG rickettsial species of unknown pathogenicity are *R. montanensis*, *R. amblyommii*, and *Candidatus* "R. andeanae". These rickettsiae have been found in ticks that carry known pathogenic *Rickettsia* species. While frequent horizontal (infectious) transmission favors virulent microbes, vertical (inherited) transmission favors evolution of benign and mutualistic (endosymbiont) associations (Werren 1997). Certain endosymbionts, such as *R. montanensis* have been reported to be abundant in ticks in the United States (Azad and Beard 1998). This abundance in nature likely influences the ecology and epidemiology of RMSF, which shares a tick host in common with *R. montanensis*. Burgdorfer et al. reported that competition between non-pathogenic and pathogenic *Rickettsia* results in decreased vertical transmission of the pathogenic *Rickettsia* (Burgdorfer et al. 1981). This competition phenomenon is termed interference (Lane 1994). As previously mentioned, a similar interference effect has been speculated, but not confirmed, with *Candidatus* "R. andeanae" preventing *R. parkeri* infection of *A. maculatum* in Oklahoma and Kansas (Paddock et al. 2015).

Not only are SFG *Rickettsia* of unknown pathogenicity important due to their ability to alter the ecology of pathogenic *Rickettsia*, but they have the potential to emerge as pathogens themselves. This type of emergence would have a major effect on public health due to the ubiquitous nature of these organisms in their tick hosts. For example, *R. amblyommii* and *R. montanensis*, symbionts of *A. americanum* and *D. variabilis*, respectively, have been implicated

as suspected pathogens based on finding DNA of these organisms in ticks that were feeding on humans that developed a rash (Billeter et al. 2007, McQuiston et al. 2012). However, these studies did not confirm that the patients were infected with these organisms, since samples from the lesions of the patients were not collected for rickettsial culture or PCR. In future cases, in order to definitely determine if these organisms are indeed human pathogens, it is imperative that inoculation site samples be collected for DNA detection or rickettsial culture. Lastly, another application of studying these bacteria of unknown pathogenicity is that, by comparing non-pathogen to pathogen, there is the potential to discover factors that are necessary for vector transmission to the host and ability to cause disease, which may lead to novel transmission-blocking preventatives.

1.2. *Amblyomma maculatum*

The Gulf Coast tick, *Amblyomma maculatum*, is an important arthropod due to its impact on human and animal health both directly by inducing disease through biting vertebrate hosts and indirectly by transmitting pathogens. This tick species belongs to the phylum Arthropoda, the class Arachnida, the order Acari, and the family Ixodidae. The original description of this hard, ornate tick with long mouthparts was recorded by Carl Ludwig Koch in 1844 based on ticks collected in the Carolinas (Koch 1844). However, it wasn't until 1912 that the pathology caused by these ticks feeding on cattle was documented (Hooker et al. 1912). Several years later, studies by R. R. Parker were the first to document an organism isolated from *A. maculatum* that when inoculated in guinea pigs resulted in a mild self-limiting fever and scrotal reaction (Parker et al. 1939). It wasn't until 2004 that *R. parkeri* rickettsiosis was described in a human patient after *A. maculatum* infestation (Paddock et al. 2004). The following sections will discuss the habitat and life cycle of *A. maculatum* and detail its medical and veterinary importance.

1.2.1. Life Cycle and Distribution

A. maculatum is a three host tick that requires feeding on three separate hosts in order to get nutrients to progress to the next life stage and complete its life cycle. In the natural setting, larvae and nymphs are typically found on small rodents and small birds, with nymphs and primarily adults feeding on larger animals such as large carnivores, ruminants, horses, or humans (Teel et al. 2010). However, a recent report demonstrated that similar to *A. americanum*, larval *A. maculatum* will feed on humans in an experimental setting (Portugal and Goddard 2015). The longevity of unfed ticks is variable depending on the life stage and environmental conditions with adults surviving twice as long as nymphs and up to four times as long as larvae when maintained at 27°C and a constant relative humidity (Teel et al. 2010). Once attached to the host, females take 8-21 days to fully engorge and drop off the host (Hixson 1940, Drummond and Whetstone 1970). It generally takes engorged females 4-9 days to oviposit, although that period may be prolonged in low temperatures (Hixson 1940, Drummond and Whetstone 1970, Wright 1971). A single engorged female produces an average of greater than 8,000 eggs with egg production peaking during the first week of oviposition and lasting up to 26 days (Hooker et al. 1912, Bishopp and Hixson 1936, Hixson 1940, Wright 1971). The incubation time of eggs before eclosion is also variable depending on environmental conditions, but is on average 30-60 days (Teel et al. 2010). Larvae engorge within a few days after attachment to the vertebrate host and molt into nymphs after 11-17 days (Hooker et al. 1912, Hixson 1940, Koch and Hair 1975). Nymphs engorge in 5-8 days after attachment to their vertebrate host and molt to adults within an average of 24-38 days, with longer molting times in adverse environments (Koch and Hair 1975).

The historical range of *A. maculatum* in the United States was limited to 150 miles of land lining the Gulf Coast states (Figure 1.11) (Hooker et al. 1912, Bishopp and Hixson 1936, Cooley and Kohls 1944, Bishopp and Trembley 1945, Paddock and Goddard 2015). However, in the past 50 years that range has extended greater than 250 miles inland, extending into land locked states (Figure 1.12) (Semtner and Hair 1973, Goddard and Paddock 2005, Goddard 2007, Teel et al. 2010, Trout et al. 2010, Pagac et al. 2014, Paddock and Goddard 2015). It was originally postulated that the narrow range of *A. maculatum* was due to its requirement for high humidity and temperature (Bishopp and Hixson 1936). However, this tick is also well adapted to drier, open grasslands and prairies within its expanded range, which is attributed to its low transpiration rate and ability to conserve moisture (Yoder et al. 2008). It is also likely that the movement of wildlife and domestic vertebrate hosts due to anthropogenic activities have contributed to this expanded geographical range (Paddock and Goddard 2015). Examples of these activities include the relocation of cattle from the Gulf Coast to the prairies of the Midwest and the introduction of free-ranging feral swine to the southeastern United States that serve as hosts for Gulf Coast ticks (Semtner and Hair 1973, Paddock and Goddard 2015). Furthermore, conservation efforts in the southeastern United States in the 20th century have resulted in a dramatic increase in populations of the white-tailed deer, another preferred host for *A. maculatum* (Paddock and Yabsley 2007, Teel et al. 2010, Paddock and Goddard 2015). Lastly, the increasing populations of migratory birds may play a role in the relocation of immature *A. maculatum* to new geographical ranges (Florin et al. 2014, Paddock and Goddard 2015). The habitats for these birds and the ticks that they harbor have grown in recent years due to prescribed burning of forests (Madden et al. 1999, Wilcox and Giuliano 2011). This phenomenon occurred in Ft. Campbell, an army base on the border of Kentucky and Tennessee, where

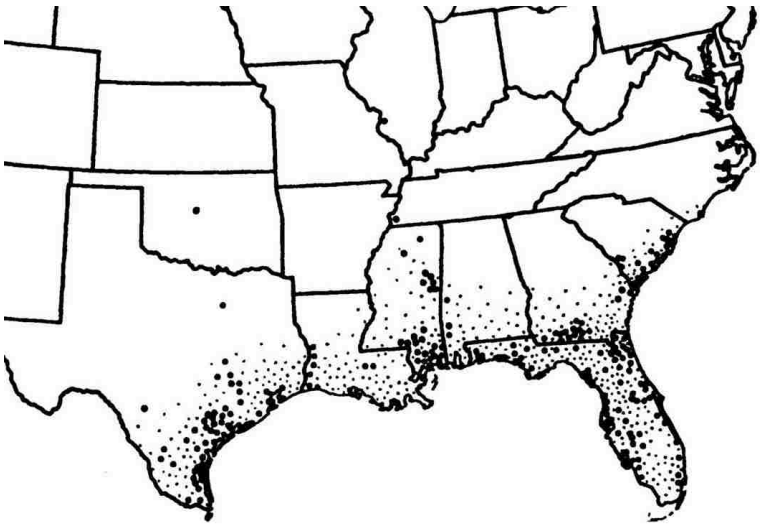

Figure 1.11. A distribution map of *A. maculatum* in the United States for 1945. This map was determined using tick collection data from the United States Bureau of Entomology and Plant Quarantine (Bishopp and Trembley 1945). Large dots indicate specific sites from which Gulf Coast ticks were collected, and small dots represent the probable distribution of the Gulf Coast tick, determined by the authors (Paddock and Goddard 2015).

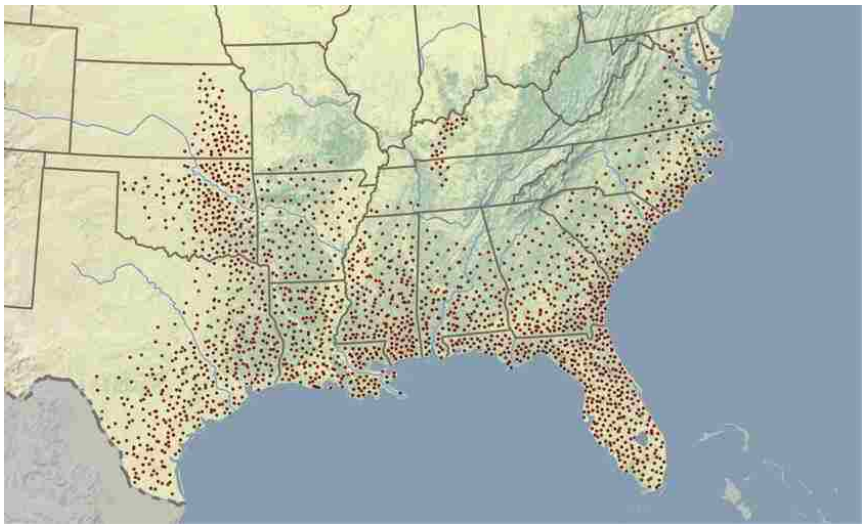

Figure 1.12. An estimated distribution of *A. maculatum* in the United States for 2014 that interpolates contemporary data (Wilson and Baker 1972, Goddard and Norment 1983, Harrison et al. 1997, Clark et al. 1998, Williams et al. 1999, Reeves et al. 2002, Barker et al. 2004, Goddard and Paddock 2005, Cohen et al. 2009, Teel et al. 2010, Trout et al. 2010, Brown et al. 2011, Fornadel et al. 2011, Varela-Stokes et al. 2011, Wright et al. 2011, Florin et al. 2014, Pagac et al. 2014). The range is shown as dark red stippling against a physical geographical base map. Loose stippling along borders represent areas where distribution and abundance may be expected to vary annually. The colors of the relief topographic map depict modern land cover conditions (naturalearthdata.com). Map courtesy of R. Ryan Lash, Centers for Disease Control and Prevention, Atlanta, GA (Paddock and Goddard 2015).

prescribed burns restored the breeding habitats for grassland birds which led to the establishment of *A. maculatum* populations in an area where they previously had not been identified (Hunter et al. 2001, Jiang et al. 2012, Pagac et al. 2014, Paddock and Goddard 2015). The expanding range of this tick has tremendous implications on tick-borne disease due to the medical and veterinary importance of this tick as will be discussed in the following section.

1.2.2. Medical and Veterinary Importance

While the major impact of the Gulf Coast tick on human health, as previously discussed, is due to its ability to transmit *R. parkeri*, bites of this tick may also be a nuisance to its human hosts and can also lead to tick paralysis (Paffenbarger 1951, Espinoza-Gomez et al. 2011, Paddock and Goddard 2015). There have been at least two reports of tick paralysis in human patients caused by *A. maculatum* with neurological deficits, such as paresis/paralysis and ataxia which resolved upon tick removal (Paffenbarger 1951, Espinoza-Gomez et al. 2011). In addition to *R. parkeri*, other human pathogens have rarely been found in *A. maculatum*, including *Ehrlichia chaffeensis* and *R. felis*, however the role of this tick in the transmission of these disease-causing agents is unknown (Williamson et al. 2010, Jiang et al. 2012, Paddock and Goddard 2015).

In addition to its medical importance, *A. maculatum* can cause dramatic, life threatening disease in other mammalian hosts including several domestic animal species. Infestations by *A. maculatum* on cattle can lead to a significant negative economic impact due to weight loss or failure to gain weight (Gladney et al. 1977, Stacey et al. 1978, Williams et al. 1978). Additionally, the open wounds caused by these arthropods can provide an ideal environment for the development of myiasis (Bishopp and Hixson 1936). In fact, prior to its eradication from the United States, as many as 40-80% of infestations by the primary screwworm, *Cochliomyia*

hominivorax, in livestock were secondary to feeding by Gulf Coast ticks (Paddock and Goddard 2015). Furthermore, due to their long mouthparts and preference for feeding on the ears of large mammals, infestations by *A. maculatum* can result in a condition called "gotch ear", where the pinna is thickened and curved due to extensive dermatitis and edema that eventually leads to destruction of the supporting cartilage and drooping of the ears (Figure 1.13) (Bishopp and Hixson 1936, Paddock and Goddard 2015). The pathophysiology of this condition is unknown, but the combination of the annoyance of the ticks feeding as well a buildup of exudate from the bite site causes the affected animals to rub their ears exacerbating their disease (Gladney et al. 1977). Gotch ear has been reported primarily in cattle as well as horses, mules, sheep, and goats (Bishopp and Trembley 1945, Drummond and Whetstone 1970, Gladney 1976, Ivey et al. 1978, Williams et al. 1978, Byford et al. 1992, Edwards 2011).

Figure 1.13. Gotch ear in cattle. The images depict the drooped and stiffened appearance that results from attachment and feeding by multiple adult *A. maculatum* to the inner surface of the ear. The irritation caused by attached *A. maculatum* ticks may result in generalized edema, inflammation, and proteinaceous exudates and eventually lead to destruction of the supporting cartilage (adapted from (Paddock and Goddard 2015)).

A. maculatum is also the primary vector, and the only definitive host for the protozoa *Hepatozoon americanum*, the causative agent of American canine hepatozoonosis (Ewing et al. 2002). This hepatozoonosis is a frequently fatal disease of domestic dogs is characterized by fever, lethargy, and muscle wasting (Ewing and Panciera 2003). Laboratory findings in these animals include a marked mature neutrophilia, hypoglycemia, hypoalbuminemia, and elevated levels of serum alkaline phosphatase (Ewing and Panciera 2003). Canines are accidental hosts that are infected by either ingesting ticks that contain oocysts or infected mice or rabbits that contain tissue cystozoites (Ewing and Panciera 2003, Johnson et al. 2008). Once inside the host, the organism encysts in muscle and then undergoes merogony to release large numbers of merozoites inciting marked pyogranulomatous myositis (Ewing and Panciera 2003, Paddock and Goddard 2015). Macrophages engulf these merozoites, which then undergo gametogeny to form gamonts. These gamonts can be found in low numbers of circulating monocytes and when found are diagnostic for this disease (Ewing and Panciera 2003, Paddock and Goddard 2015). Treatment of American canine hepatozoonosis relies on a combination of antiprotozoals and non-steroidal anti-inflammatory drugs for pain relief (Ewing and Panciera 2003). A prolonged therapeutic course may be required as relapse is likely to occur (Macintire et al. 2001).

In addition to its role as a vector of animal and human pathogens, certain wild-caught populations of *A. maculatum* also harbor a rickettsial species of unknown pathogenicity, *Candidatus* "Rickettsia andeanae" (Blair et al. 2004, Jiang et al. 2005, Paddock et al. 2010, Fornadel et al. 2011, Varela-Stokes et al. 2011, Wright et al. 2011, Ferrari et al. 2012, Jiang et al. 2012, Luce-Fedrow et al. 2012, Ferrari et al. 2013, Flores-Mendoza et al. 2013, Leydet and Liang 2013, Nadolny et al. 2014, Paddock et al. 2015). This bacterial species has been identified via immunohistochemistry in low numbers in the skin of mice which were infested by *A.*

maculatum (Grasperge et al. 2014). However, no pathology was associated with inoculation of these organisms (Grasperge et al. 2014). Therefore, future study to identify factors that determine rickettsial pathogenicity could be performed by comparing rickettsial inoculation by ticks infected with *Candidatus* "Rickettsia andeanae" to those infected by *R. parkeri*. These studies would investigate whether the differences in pathogenicity are due to intrinsic bacterial properties or whether the pathogenicity is determined by the amount of rickettsiae inoculated by ticks. Similarly, other future investigations of tick-borne pathogens, such as *R. parkeri* should involve evaluation of the role that the vector plays in rickettsial transmission and subsequent disease progression.

1.3. Mammalian Immune Response to SFG *Rickettsia*

Studies that utilize animal models are indispensable to infectious disease research as they are essential to elucidate the pathogenesis and immune response for a particular pathogen in order to develop potential therapeutics, and vaccine strategies. The ideal goal of designing an animal model should be to replicate human disease via the natural route of infection in an easy, highly reproducible and cost-effective manner. This section of the review will highlight some of the models that have been developed to study SFG rickettsioses, as well as the advantages and disadvantages of each of these models. The insights gained through these studies with regards to innate and adaptive immunity will then be discussed.

1.3.1. Animal Models of SFG *Rickettsia*

Within the SFG of *Rickettsia* most of the studies using animal models have been done with *R. rickettsii* and *R. conorii* and some recent work on *R. parkeri*. Several animal species have been used to model different aspects of SFG rickettsial disease via a variety of inoculation routes. As previously mentioned, the preliminary research in the field of rickettsiology carried

out by Ricketts in the early 20th century utilized guinea pigs as a model (Ricketts 1906a). When inoculated intraperitoneally with *R. rickettsii*, these animals developed fever, macular rash, scrotal swelling and bruising, and died by two weeks post-inoculation (Ricketts 1906a). These results have since been recapitulated with the addition of the characterization of the typical vasculitis and detection of rickettsiae within lesions (Walker et al. 1977). Similarly, *R. conorii* causes a fever and scrotal edema when inoculated intraperitoneally in guinea pigs, but, did not cause disease when injected subcutaneously (Hass and Pinkerton 1936). While intradermal inoculation of *R. conorii* results in the formation of a local cutaneous inoculation site eschars and rickettsemia in guinea pigs, this more natural inoculation route does not induce a fever or other signs of illness (Walker et al. 1992, La Scola et al. 2009). Similarly, guinea pigs also developed eschars without systemic illness when intradermally inoculated with 15 other rickettsial species, and 13 of these species had been previously associated with eschars in humans (La Scola et al. 2009). While these studies demonstrate that guinea pigs can be susceptible to some SFG rickettsial species, other models are necessary that do not rely on the intraperitoneal route of infection to induce systemic disease. Rhesus macaques have also been used to model RMSF and are susceptible to intravenous inoculation, developing a fever, erythema, cyanosis, and rash as well as a high fatality rate when inoculated with high doses. Fatality in these animals is associated with thrombocytopenia, elevated fibrin degradation products, and prolonged clotting times (Ricketts 1906a, Mosher et al. 1977). While these animals may be better models for human disease, their cost and restrictions on availability limit their widespread use as animal models. Many other animals including ferrets, gerbils, hamsters, rabbits, cotton rats, miniature pigs, and sheep have been shown to be resistant to SFG rickettsioses via intraperitoneal and subcutaneous inoculations (Sammons et al. 1977). Additionally, cotton rats have been shown to develop a

transient rickettsemia after intracardiac or intraperitoneal inoculation with *R. rickettsii*, but remain asymptomatic (Shirai et al. 1967). Pine voles are susceptible to *R. rickettsii* via intraperitoneal inoculation with high morbidity and mortality (Eremeeva et al. 2003). However, the lack of available reagents for this species limits the usefulness of this model.

Due to the previously described limitations on animal susceptibility, availability, ease of use, and artificial route of inoculation in the above models, there is a need to explore other models, which has led to a number of studies evaluating various inbred mouse strains as models for SFG rickettsioses. The biggest advantages to using these animals are the widespread availability, ease of use, reproducibility of disease due to similar genetic makeup, and large number of reagents available for use with these animals. However, similar to other rodents, the various strains of mice have varying susceptibility to SFG *Rickettsia* and frequently rely on artificial routes of inoculation in order to produce disease. For example, in a study of *R. conorii* in 20 inbred mouse strains, only C3H/HeJ mice proved to be susceptible, where 10 plaque forming units inoculated intraperitoneally resulted in fatality 50% of the time (Eisemann et al. 1984). However, subcutaneous inoculations in these animals did not result in death even at doses of up to $5x10^{10}$ organisms (Eisemann et al. 1984). C3H/HeN mice have also been shown to be susceptible to high doses of *R. conorii* inoculated intravenously developing endothelial infection, interstitial pneumonia, and hepatic granulomas (Walker et al. 1994). This model still avoids the natural route of inoculation via tick inoculation. However, lesions seen in human cases of SFG rickettsioses are consistently reproduced, and therefore this model has been used subsequently for immunology studies as will be described in the following sections (Feng et al. 1994, Billings et al. 2001, Valbuena et al. 2003, Rydkina et al. 2004, Valbuena and Walker 2004, Valbuena and Walker 2005, Fang et al. 2009, Jordan et al. 2009, Riley et al. 2015, Riley et al. 2016).

While all of the previously mentioned models utilize the artificial route of needle inoculation of SFG *Rickettsia*, two recent animal models of RMSF have been established where dogs were infected with two different strains of *R. rickettsii* via experimentally infected ticks (Piranda et al. 2008, Levin et al. 2014). Dogs exposed to a Brazilian strain of *R. rickettsii* via infected *A. aureolatum* ticks developed fever, lethargy, anorexia, ocular lesions, thrombocytopenia, anemia, rickettsemia and anti-*Rickettsia* antibodies post-inoculation and recovered from their illness without treatment (Piranda et al. 2008). Dogs that were inoculated with the American strain of *R. rickettsii* via infected *D. variabilis* also developed fever, lethargy, anorexia, ocular lesions, anemia, and thrombocytopenia in addition to mucosal petechiae, tremors, skin rash, and inflammatory leukograms (Levin et al. 2014). One dog was euthanized after exhibiting convulsions, and typical vasculitis was demonstrated in multiple organs (Levin et al. 2014). The remainder of the dogs recovered either without treatment or after treatment with doxycycline, although one dog required two rounds of treatment after its clinical signs returned two weeks after completion of the initial treatment (Levin et al. 2014).

In addition to the models described above for *R. conorii* and *R. rickettsii*, there are a few reports describing animal models for *R. parkeri*. The original research on *R. parkeri,* well before it was discovered to be a pathogen, was performed in guinea pigs via intraperitoneal inoculation, and inoculated animals were either asymptomatic or developed a mild fever and swelling and erythema of the scrotum (Parker et al. 1939). Opossums inoculated intraperitoneally with *R. parkeri* are asymptomatic, but develop transient rickettsemia and can transmit this infection to uninfected ticks (Horta et al. 2010). Additionally, *R. parkeri* persists in the tissues of cotton rats after subcutaneous inoculation; however no pathology or clinical signs were reported in response to these inoculations (Moraru et al. 2013). The most promising animal model for *R. parkeri*

rickettsiosis is the C3H/HeJ mouse, which develops facial edema, splenomegaly, and high tissue rickettsial loads in response to intravenous inoculation (Grasperge et al. 2012). Furthermore, eschar-like lesions characterized histologically by marked vasculitis were noted when these animals were inoculated intradermally in the tail (Figure 1.14) (Grasperge et al. 2012). *A. maculatum* feeding after intradermal inoculation in these mice exacerbated cutaneous pathology with increased rickettsial load, demonstrating the role of the vector in *R. parkeri* rickettsiosis (Grasperge et al. 2014). While these mice are valuable to model the lesions of *R. parkeri* rickettsiosis, they have a mutation in TLR4, which is needed for stimulation of dendritic cells and activation of the cell-mediated immune response necessary for anti-rickettsial immunity (Hoshino et al. 1999, Jordan et al. 2008). Therefore, in order to evaluate the immune response to *R. parkeri* rickettsiosis, a different, immunocompetent, animal model would be needed.

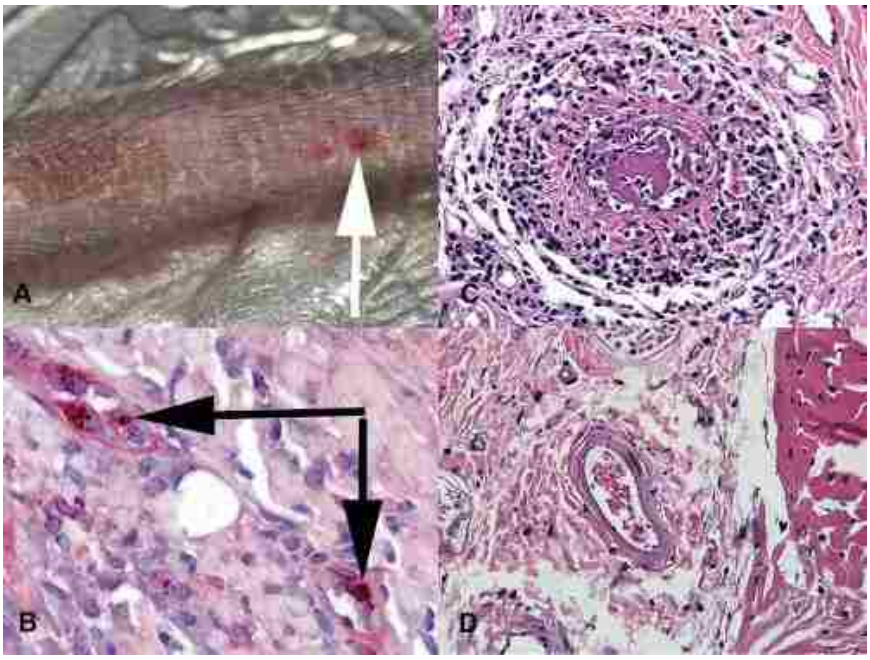

Figure 1.14. Gross histopathology of eschar-like lesions in C3H/HeJ mice following intradermal inoculation of *R. parkeri* at 27 dpi. (A) Gross lesions associated with inoculation site in *R. parkeri*-infected C3H/HeJ mouse (white arrow). (B) Immunohistochemistry displaying positive staining (black arrows) in the cytoplasm of endothelial cells and macrophages of *R. parkeri*-infected C3H/HeJ mice. (C) Marked vasculitis in *R. parkeri*-infected C3H/HeJ mouse. (D) Uninfected C3H/HeJ mouse histopathology (for comparison) (Grasperge et al. 2012).

1.3.2. Innate Immune Response to SFG *Rickettsia*

Studies both of human cases of rickettsioses and those utilizing the animal models described in the preceding section have demonstrated that SFG *Rickettsia* target endothelial cells as well as macrophages and dendritic cells leading to an anti-rickettsial immune response and resulting in the characteristic vasculitis seen with rickettsial disease (Walker and Ismail 2008, Mansueto et al. 2012). Both the innate and adaptive arms of the immune response play a role in anti-rickettsial immunity as summarized in this and the following section (Figure 1.15) (Walker and Ismail 2008). The innate immune response is initiated through the interaction of pathogens with pattern-recognition receptors such as the TLRs (Mansueto et al. 2012, Sahni et al. 2013). TLR pathway activation results in activation of NF-κB and the production of numerous pro-inflammatory cytokines and the acute phase inflammatory response (Sahni et al. 2013). The acute phase response has been demonstrated in humans with Mediterranean spotted fever (MSF) caused by *R. conorii* as indicated by elevations in serum inflammatory cytokines such as interferon γ (IFN γ), interleukin-6 (IL-6), and tumor necrosis factor α (TNFα), as well as the acute phase protein, C-reactive protein during the first 1-2 weeks of infection (Mansueto et al. 1994, Vitale et al. 2001). Furthermore, increased expression of IFN γ and TNFα has been noted in biopsies of skin lesions from patients with MSF and was correlated with mild to moderate disease as opposed to severe disease; however, a cause and effect relationship was not demonstrated (de Sousa et al. 2007). As previously discussed the importance of TLR4 in rickettsial infection has been suggested by the fact that C3H/HeJ mice, that have a mutation in TLR4, are more susceptible to various SFG rickettsioses than other inbred mouse strains (Jordan et al. 2008, Grasperge et al. 2012). Furthermore, studies of *R. conorii* infection in TLR4-deficient mice showed that TLR4 activation of dendritic cells via rickettsial LPS was important for the

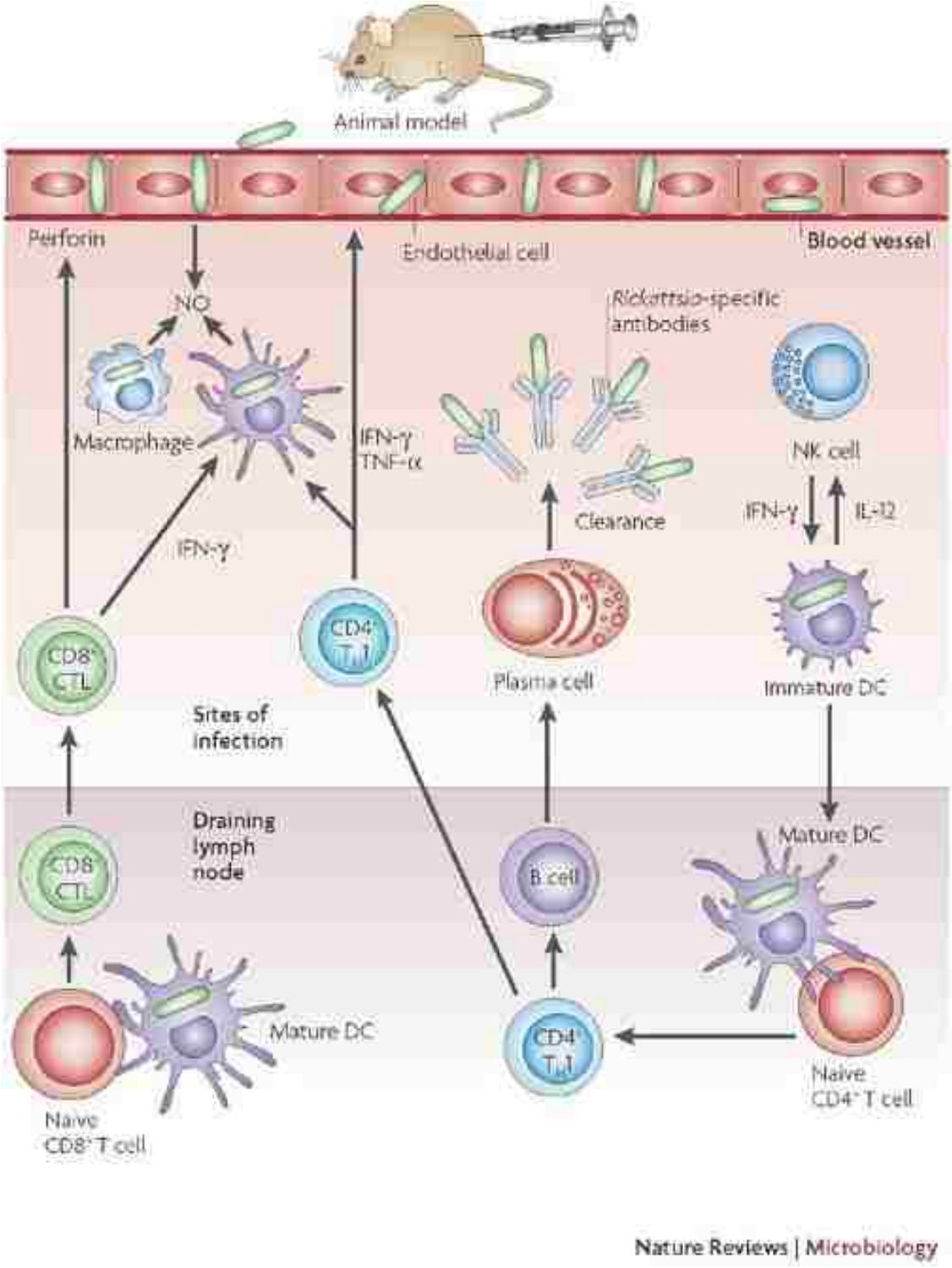

Figure 1.15. Model of protective immunity in rickettsial infection (Walker and Ismail 2008).

subsequent activation of natural killer (NK) cells and production of large quantities of IFNγ (Jordan et al. 2009). The importance of TLR4 signaling through MyD88 in rickettsial immunity was also recently demonstrated as MyD88 knockout mice have decreased ability to clear rickettsial infection with lower inflammatory infiltrates and expression of IFNγ, IL-12, IL-6 and granulocyte colony stimulating factor (G-CSF) (Bechelli et al. 2016). NK cells are also important in the acute innate immune response to SFG *Rickettsia*, as depletion of these cells results in enhanced susceptibility to *R. conorii* infection as well as a decrease in the serum concentrations of IFNγ and IL-12 (Billings et al. 2001).

Dendritic cells, in addition to activating NK cells, are also infected by *Rickettsia*, which are found both within the cytoplasm and endosomes of these cells indicating that they can present antigen via both major histocompatibility complex (MHC) class I and II (Fang et al. 2007). In *R. conorii*-resistant mice, infected dendritic cells produce cytokines such as IL-12 that stimulate a protective Th1 immune response and production of IFNγ by CD4+ T cells as opposed to the proliferation of Foxp3+ regulatory T cells seen in susceptible mice (Fang et al. 2007). The production of cytokines such as IFNγ and TNFα are important to the activation of phagocytic cells as depletion of these cytokines in mice infected with a sublethal dose of *R. conorii* resulted in mortality and overwhelming rickettsial load due to impaired nitric oxide (NO) production by phagocytes (Feng et al. 1994). Once infected and stimulated by cytokines, human endothelial cells, macrophages, and hepatocytes are capable of killing *R. conorii in vitro* via a variety of mechanisms including the induction of NO, oxidative burst, production of hydrogen peroxide, and/or tryptophan degradation (Feng and Walker 2000). Using animal models, it has been determined that a variety of cell types such as NK cells, macrophages, and dendritic cells, which are activated via receptors such as TLRs and various stimulatory cytokines play an important

role in the initiation of the anti-rickettsial immune response. This innate response is not only important in the acute phase of infection, but also drives the formation of the specific, long-term adaptive immune response.

1.3.3. Adaptive Immune Response to SFG *Rickettsia*

While the innate immune response is essential for the initial response against SFG *Rickettsia*, studies of human cases of rickettsiosis and animals models have also demonstrated the important role of the adaptive immune system in this response (Walker and Ismail 2008, Mansueto et al. 2012, Sahni et al. 2013). Both CD4+ helper T cells and CD8+ cytotoxic T cells have been found in perivascular infiltrates in *R. conorii* eschars (Herrero-Herrero et al. 1987). As indicated earlier, the production of Th1 cytokines such as TNFα and IFNγ by CD4+ T helper cells is crucial for anti-rickettsial immunity (Fang et al. 2007). Cytotoxic CD8+ T cells are also integral to the anti-rickettsial response via secretion of IFNγ as well as MHC class I-mediated killing of *Rickettsia* infected cells via secretion of cytolytic enzymes like perforin, and have been shown to reduce tissue rickettsial loads in IFNγ knockout mice (Walker et al. 2001, Walker and Ismail 2008). In addition to cell-mediated immunity, the antibody response to *Rickettsia* has been shown to be important to combat disease. Antibodies to *R. conorii* OmpA and OmpB protected SCID mice from *R. conorii* infection as opposed to those receiving anti-LPS antibodies or Fab fragments of antibody (Feng et al. 2004a). Furthermore, *in vitro* studies in endothelial and macrophage-like cell lines demonstrated that this Fc-dependent effect was due to opsonization and enhanced killing of *R. conorii* within phagolysosomes (Feng et al. 2004b). In opposition to the protective role of the cell-mediated immune response, CD4+ CD25+ Foxp3- regulatory T cells have been shown to play an immunosuppressive role (Fang et al. 2009). Higher numbers of these cells were found in lethal *R. conorii* infection, and splenocytes from these mice produced

lower concentrations of protective Th1 cytokines such as IL-2 and IFNγ and higher concentrations of the inhibitory cytokine, IL-10 (Fang et al. 2009). While the studies described above have detailed several aspects of the anti-rickettsial immune response, it should be noted that most of these studies rely on intravenous needle inoculation of the organisms, as opposed to the natural route of tick inoculation. It is important to consider the role of the vector in SFG *Rickettsia* immunity, because, as described in the next section, tick feeding and salivary components have been shown to significantly alter several aspects of both the innate and adaptive vertebrate host immune response.

1.4. Tick-Host Interactions

As stated previously, *A. maculatum* and other hard ticks require an uninterrupted blood meal of several days duration in order to mature from one life stage to the next. Therefore, these arthropods must be able to counteract host defenses such as the host pain and itch response, hemostasis, wound healing, and the cellular and secreted components of the host immune response. As such, their salivary components have been investigated as therapeutics that counteract these processes. Interestingly, these molecules are also gaining interest in the field of oncology research as anti-tumor drugs due to their cytotoxic and cytolytic properties that act against various cell types as well as anti-angiogenic properties (reviewed in (Sousa et al. 2015)). Due to the importance of ticks as vectors of several infectious organisms of bacterial, viral, and protozoal origin, there also has been significant research on the immunomodulatory properties of their saliva and salivary gland extracts (reviewed in (Kotal et al. 2015)). Additionally, numerous studies have been performed to identify the various immunomodulatory components in the salivary glands of a number of tick species including *A. maculatum* via high-throughput transcriptomics and proteomics (reviewed in (Chmelar et al. 2015)). These studies can also be

performed to compare salivary gland composition in various stages of development/feeding as well as the effect of pathogen infection on these components. Using these techniques researchers may be able to identify specific molecules that are only expressed by ticks during pathogen transmission and discover potential targets of transmission-blocking therapeutics. The following section will describe the current knowledge of how tick salivary components modulate many aspects of the mammalian host immune response followed by a discussion of the studies that have shown that tick salivary components or feeding can augment pathogen infection.

1.4.1. Tick Immunomodulation

As ticks attach to a vertebrate host, they encounter several immune cells as well as secreted molecules that must be counteracted in order to feed to repletion. Complement is an example of one of these non-cellular factors. The saliva of various species of *Ixodes* ticks as well as specific factors such as Isac, IRAC, and Salp20 have been shown to inhibit the formation and inactivate the end products of the alternative complement pathway (Ribeiro and Spielman 1986, Ribeiro 1987, Valenzuela et al. 2000, Daix et al. 2007, Tyson et al. 2007, Hourcade et al. 2016). Furthermore, as reviewed in Kotal et. al. (2015), tick saliva or salivary gland extract (SGE) from various tick species as well as the presence of feeding ticks have been shown to alter the function of a variety of host immune cells involved in the innate immune response (Table 1.6), as well as T and B lymphocytes of the adaptive immune response (Table 1.7). SGE from a variety of hard ticks has been shown to decrease NK cell activity and cytotoxicity. Saliva or SGE from several hard tick species has also been shown to inhibit neutrophil chemotaxis, adhesion, phagocytosis, and killing ability. Additionally, macrophage phagocytosis, NO and cytokine production is inhibited by the saliva and/or SGE of hard tick species. Dendritic cell maturation, differentiation, migration, proliferation, and cytokine production is inhibited by saliva collected from a number

Table 1.6. The effects of tick saliva, SGE, or tick feeding on innate immune cell populations (adapted from (Kotal et al. 2015)).

Tick	Saliva/SGE/ Feeding	Effect
NK cells		
A. variegatum	SGE	Decreased NK cell activity
Dermacentor reticulatus	SGE	Decreased NK cell activity
Haemaphysalis inermis	SGE	Decreased NK cell activity
I. ricinus	SGE	Suppression of NK cell cytotoxicity
Macrophages		
Dermacentor variabilis	Saliva	Impaired phagocytosis and altered gene expression, stimulation of migration Stimulation of PGE2 production, inhibition of cytokine production
Ixodes ricinus	SGE	Inhibition of superoxide and NO production Inhibition of phagocytosis and TNF production
Ixodes scapularis	Saliva	Inhibition of cytokine production Inhibition of NO production
Rhipicephalus appendiculatus	SGE	Inhibition of cytokine and NO production
Rhipicephalus microplus	SGE	Altered surface molecule expression, inhibition of cytokine production
Rhipicephalus sanguineus	Saliva	Inhibition of NO production
Dendritic cells		
Amblyomma cajennense	Saliva	Inhibited maturation and differentiation; reduced migration due to decreased expression of receptors; polarization towards Th2 cytokines
I. ricinus	Saliva	Inhibited maturation, migration and antigen presentation; blocked Th1 and Th17 polarization Inhibited proliferation, phagocytosis and cytokine production Impaired maturation and cytokine production Inhibition of signaling pathways
I. scapularis	Saliva	Inhibition of proliferation and cytokine production
Rh. Sanguineus	Saliva	Reduced migration, maturation and cytokine production
Eosinophils		
Soft and hard ticks	Feeding	Increased amount of eosinophils in feeding cavity
Hard ticks	SGE	Inhibition of attraction to the feeding site
I. ricinus	Saliva	Basophil activation via MCP-1 released from splenocytes

(Table 1.6 continued)

Tick	Saliva/SGE/ Feeding	Effect
Basophils		
Amblyomma cajennense	Feeding	Increased amount of basophils in feeding cavity
Amblyomma dubitatum	Feeding	Increased amount of basophils in feeding cavity
Neutrophils		
Soft and hard ticks	SGE	Anti-IL-8 activity
Amblyomma americanum	SGE	Altered dynamics of chemokine activity
I. ricinus	Saliva	Decrease in ROS production
I. scapularis	Saliva	Inhibition of granule release, infiltration, phagocytosis Reduced adhesion of polymorphonuclear leukocytes
Rh. Appendiculatus	SGE	Altered cytokines mRNA production by peripheral blood leukocytes
Rh. Microplus	SGE	Inhibition of phagocytosis

of hard ticks. Conversely, feeding of ticks has been documented to result in an infiltrate of basophils and eosinophils at the cutaneous tick bite site. Lastly, the saliva, SGE, and/or feeding of several Ixodid tick species has been shown to inhibit B and T lymphocyte proliferation and suppress their responsiveness, as well as inhibit the production of Th1 cytokines (such as IL-2, IFNγ, and IL-12), while increasing the production of Th2 cytokines (such as IL-4 and IL-10). The major limitation of these studies is that with few exceptions, the immune cells studied were either derived from bone marrow, spleen, or peripheral blood, which may not function similarly to those found in the skin. Furthermore, SGE, which is produced by sonication of whole salivary glands removed from ticks, likely has a different composition than what is inoculated during tick feeding. Even saliva, which is collected only at one time point during feeding, may not completely represent the various factors inoculated during the entire tick feeding process. In fact, a study of salivary glands collected from *A. americanum* determined that there was a marked difference in the expression of transcripts of secretory proteins at different time points during tick feeding (Karim and Ribeiro 2015).

Table 1.7. The effects of tick saliva, SGE, or tick feeding on lymphocytes (adapted from (Kotal et al. 2015)).

Tick	Saliva/SGE/ Feeding	Effect
Soft and hard ticks	Saliva, SGE	Polarization of the immune response towards Th2 via cytokines
Amblyomma variegatum	SGE	Inhibition of lymphocyte proliferation
Dermacentor andersoni	SGE	Reduced T cells proliferation Reduced Th1 cytokine production
	Saliva, SGE, feeding	Inhibition of integrin expression
	SGE, feeding	Increased IL-4 and IL-10 levels
Haemaphysalis bispinosa	Feeding	Reduction in T lymphocyte count and proliferation, increased CD4 +/CD8 + ratio
Hyalomma anatolicum anatolicum	Feeding	Reduction in T lymphocyte count and proliferation, increased CD4 +/CD8 + ratio, increase in circulating B lymphocyte count
I. ricinus	SGE	Inhibition of lymphocyte proliferation Suppression of B cell proliferation, inhibition of IL-10 production, reduction of markers on the surface of T and B cells
	Saliva	Inhibition of T cell proliferation Induction of Th2 differentiation of CD4 + T cells via dendritic cells
	Feeding	Increased CD4 +/CD8 + ratio Inhibited proliferation and responsiveness Reduced amount of specific Ig against antigen, no change in total Ig amount
I. scapularis	Saliva	Inhibition of IL-2 production by T cells, inhibition of splenic T cell proliferation
	Feeding	Inhibition of Th17 immunity, priming of a mixed Th1/Th2 response during secondary infestation
	SGE, feeding	Increased IL-4 levels
Rh. appendiculatus	SGE	Inhibition of lymphocyte proliferation
Rh. microplus	Feeding	Decreased T and B lymphocyte percentage among PBLs
	Saliva	Decreased PBL responsiveness to phytohemagglutinin Inhibition of the blastogenic response of mononuclear cells
Rh. sanguineus	Feeding	Suppressed response to mitogens
	Saliva	Suppressed response to mitogens
	SGE	Suppressed Ig production by PBL

1.4.2. Saliva Enhanced Transmission of Tick-borne Pathogens

It is no surprise that with a profound effect on the vertebrate host immune response, tick feeding has also been associated with facilitation of pathogen transmission and subsequent infection. This effect has been reported for the transmission of a variety of bacteria, protozoa, and viruses by a number of hard tick species (reviewed in (Kazimirova and Stibraniova 2013)). The majority of this work has been performed in *Ixodes* spp. ticks and the pathogens that they transmit including *Borrelia* spp., *Francisella tularensis, Anaplasma phagocytophilum* and tick-borne encephalitis virus (reviewed in (Kazimirova and Stibraniova 2013)). Inoculation with SGE, saliva, or specific salivary molecules (ex. Salps) from these ticks increases transmission and/or infectivity to co-feeding ticks secondary to host immunomodulation (reviewed in (Kazimirova and Stibraniova 2013)). A similar effect has been noted with the SGE of *Rhipicephalus appendiculatus* and transmission of both Thogoto virus and the protozoa *Theileria parva* (Jones et al. 1989, Shaw et al. 1993). Again, it is important to note that most of these studies rely on the inoculation of saliva or SGE and do not investigate the role of the feeding tick on transmission. There are a few studies that have investigated how tick feeding augments pathogen transmission including studies on SFG *Rickettsia* and their tick vectors. One such experiment, as was previously described, demonstrated that *A. maculatum* feeding after *R. parkeri* inoculation resulted in increased rickettsial load and pathology as compared to *R. parkeri* inoculation alone (Grasperge et al. 2014). Another study found a decrease in RNA levels of IL-1 and NF-κB in the lungs of mice inoculated with *R. conorii* and infested with *Rh. sanguineus* as opposed to *R. conorii* inoculation alone (Milhano et al. 2015). However, the significance of this finding is unknown because tick infestation did not alter rickettsial load or lung infiltrates.

Therefore, there is still a need for research of the immune response to *Rickettsia* and the immunomodulatory role of ticks at the cutaneous inoculation site.

1.5. Summary

As presented in this review, *R. parkeri* has only relatively recently been described as a human pathogen. Therefore, there are very few studies evaluating the biology and the immune response incited by this organism. Furthermore, while there has been a large body of research on rickettsial immunity and tick modulation of the host immune response, there is a lack of research detailing how tick feeding at the cutaneous bite site alters the host immune response to rickettsial infection and the role that this modification plays on pathogen transmission. Therefore, two animal models were used to evaluate the effect of *A. maculatum* feeding/saliva on the vertebrate immune response and rickettsial infection. The goal of these studies was to test the overall hypothesis that if *A. maculatum* feeding plays an in immunomodulatory role in the vertebrate host, then this immunomodulation enhances infection and pathology of *R. parkeri*.

The first study utilized rhesus macaques as an immunocompetent mammalian host to evaluate intradermal *R. parkeri* inoculation in the presence and absence of tick feeding. The hypothesis was that tick feeding at the rickettsial inoculation site would alter the host response and result in increased local disease. All animals developed cutaneous eschars in response to intradermal *R. parkeri* inoculation alone with evidence of a systemic inflammatory response. Furthermore, tick feeding during inoculation resulted in larger eschars as well as exacerbation of the inflammatory response, presumably due to immunomodulatory factors in the tick saliva.

The second study was performed in immunocompentent C3H/HeN mice to evaluate the cutaneous acute innate immune response to rickettsial inoculation in the presence and absence of *A. maculatum* saliva. The hypothesis being tested was that *A. maculatum* saliva enhances *R.*

parkeri infection via downregulation of the acute cutaneous cellular and cytokine immune response. This study revealed that tick saliva inhibited the cutaneous infiltration of macrophages and neutrophils within 24 hours of rickettsial inoculation as evaluated via flow cytometry and cytological evaluation. Furthermore, cutaneous inflammatory cytokines were elevated in response to *R. parkeri* inoculation, but this response was not modulated by the addition of *A. maculatum* saliva. Taken together, these studies indicate that despite the immunosuppressive role of saliva, tick feeding itself exacerbates the local cutaneous lesions associated with the pathogen. This research highlights the role of the vector in rickettsiosis and the need for future investigations into the immunomodulatory factors inoculated by ticks during feeding.

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CHAPTER 2
AMBLYOMMA MACULATUM FEEDING AUGMENTS *RICKETTSIA PARKERI* INFECTION IN A RHESUS MACAQUE MODEL: A PILOT STUDY[1]

2.1. Introduction

Within the past fifteen years, there has been a more than four-fold increase in the number of tick-borne rickettsial disease cases in humans in the United States (Dumler 2010, Adams et al. 2014). During this time frame, *Rickettsia parkeri*, a member of the spotted fever group (SFG) of *Rickettsia* transmitted by *Amblyomma maculatum* (the Gulf coast tick), was first identified as a human pathogen (Paddock et al. 2004) with several cases reported in North and South America (Whitman et al. 2007, Paddock et al. 2008, Cragun et al. 2010, Romer et al. 2011, Portillo et al. 2013, Kaskas et al. 2014, Romer et al. 2014). Clinical signs include fever, headache, malaise, myalgia, arthralgia, formation of a maculopapular rash and multiple eschars (Whitman et al. 2007, Paddock et al. 2008, Cragun et al. 2010, Romer et al. 2011, Portillo et al. 2013, Kaskas et al. 2014, Romer et al. 2014). The eschar, along with milder symptoms, can be used to differentiate this disease from the more virulent *R. rickettsii*, which causes Rocky Mountain spotted fever (RMSF) (Paddock et al. 2004, Paddock et al. 2008). An *R. parkeri*-associated eschar is a 0.5–2 cm in diameter, crusted, non-pruritic ulcer, surrounded by an indurated, erythematous halo. These lesions are characterized histologically by extensive necrosis of the epidermis and superficial dermis and prominent lymphohistiocytic vasculitis of dermal vessels (Paddock et al. 2004, Paddock et al. 2008, Cragun et al. 2010, Kaskas et al. 2014).

Despite the recent emergence of *R. parkeri*, there have been few experimental models detailing the pathology, immune response and transmission of *R. parkeri* in mammalian hosts. A murine model has been developed in C3H/HeJ mice (Grasperge et al. 2012). Using this model, *A. maculatum* nymph feeding subsequent to intradermal injection of *R. parkeri* resulted in

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increased pathogen load and associated pathology when compared to needle inoculation alone (Grasperge et al. 2014). However, C3H/HeJ mice have a mutation in toll-like receptor 4 (TLR4) (Hoshino et al. 1999). Signaling via TLR4 is needed for stimulation of dendritic cells and activation of natural killer cells, which kill SFG *Rickettsia*-infected cells (Jordan et al. 2008). Therefore, in order to study the immune response to *R. parkeri*, an immunocompentent host would be necessary. While immunocompentent cotton rats become infected with *R. parkeri* after subcutaneous injection, they do not develop characteristic eschars (Moraru et al. 2013). Eschars formed after intradermal inoculation of *R. parkeri* in a guinea pig model; however, the effect of inoculation on other organ systems and the underlying immune reaction were not evaluated (La Scola et al. 2009). In order to model human disease and immune response, immunocompentent rhesus macaques were used in this pilot study.

As reviewed recently by Wikel (Wikel 2013), tick saliva contains substances that are capable of inhibiting a variety of cytokines, chemokines, and several other bioactive molecules. Tick saliva also has the ability to impair the function of several immune cells including natural killer cells, macrophages, neutrophils, and T and B lymphocytes (Wikel 2013). While much of this work is based on other hard tick species, salivary molecules of *Amblyomma* sp. have been shown to inhibit chemokine, natural killer cell, and dendritic cell functions (Kubes et al. 2002, Hajnicka et al. 2005, Vancova et al. 2007, Peterkova et al. 2008, Carvalho-Costa et al. 2015). It is no surprise that with this immunosuppressive ability, tick feeding has been found to enhance transmission of a variety of tick-borne pathogens including viruses (Thogotovirus and tick-borne encephalitis virus) and bacteria (*Borrelia afzelii*, *B. burgdorferi*, *B. lusitaniae*, *Anaplasma marginale*, *A. phagocytophilum*, and *Francisella tularensis*) (Nuttall and Labuda 2004, Wikel

2013). However, the effect of *A. maculatum* feeding on *R. parkeri* rickettsiosis and the immune response in a mammalian model has not been comprehensively studied.

The experiments detailed in this report were designed to reproduce disease caused by *R. parkeri* via intradermal inoculation during adult *A. maculatum* feeding in rhesus macaques as compared to two other treatments: *R. parkeri* inoculation and *A. maculatum* feeding alone. The broad hypothesis is that by modulating the host immune response, tick feeding enhances infection and pathology of pathogenic SFG *Rickettsia*. We demonstrated that tick feeding during *R. parkeri* inoculation resulted in larger areas of necrosis with delayed healing as compared to *R. parkeri* inoculation alone. Furthermore, greater neutrophilia and interleukin (IL)-6 concentrations were noted in animals inoculated during tick feeding. Lastly, in a tick + *R. parkeri* animal, rickettsial DNA was detected in a draining lymph node in the acute phase of infection and in the skin at the inoculation site in the chronic phase of infection suggesting the possibility of greater dissemination and persistence of *Rickettsia* in response to tick feeding. Taken together, these results reveal the utility of a primate model of *R. parkeri* infection and demonstrate that tick feeding can modify the pathogenesis of tick-borne rickettsiosis.

2.2. Materials and Methods

2.2.1. Tick and *Rickettsia* Preparation

A colony of *R. parkeri*-free *A. maculatum* was maintained on rodents as previously described (Troughton and Levin 2007, Grasperge et al. 2014). All animals that were used for tick-rearing purposes were housed at the Louisiana State University (LSU) Division of Laboratory Animal Medicine (DLAM) vivarium on a 12-hour light-dark cycle with ad libitum rodent feed and water. Animals were housed in social pairs or groups appropriate to the species until tick placement; at which point, they were housed individually in order to prevent partner

manipulation of tick containment devices. Larvae were fed on adult BALB/c mice (LSU DLAM, Baton Rouge, LA, USA) that were housed on wire grates over fresh water, and engorged larvae were collected twice daily as the water was changed. Nymphal and adult ticks were fed on adult Sprague-Dawley rats (LSU DLAM) or adult Hartley guinea pigs (Charles River Laboratories, Wilmington, MA, USA) within capsules fashioned from plastic 50 ml conical tubes and attached with a 3:1 tree rosin to bee wax mixture. After tick collection following feeding to repletion and dropping off of their hosts, animals were humanely euthanized with carbon-dioxide followed by cervical dislocation. Animal care and use for tick rearing purposes was approved by the Louisiana State University Institutional Animal Care and Use Committee (IACUC) (Protocol Number: 13-034).

The ticks used in this experiment were determined to be free of *R. parkeri* via DNA extraction and traditional semi-nested PCR using the 190.70p and 190.602n and 190.70p and 190.701 primer pairs for *Rickettsia ompA* as previously described (Regnery et al. 1991, Fournier et al. 1998, Pornwiroon et al. 2006, Grasperge et al. 2014). Thirty female and fifteen male adult ticks were utilized in this study. Semi-purified rickettsiae were recovered from *R. parkeri* (Portsmouth strain) (Paddock et al. 2004) passage 4 infected Vero cells (3 days post-inoculation) using the modified protocol of Weiss et al. (Weiss 1973) as previously described (Petchampai et al. 2014). Rickettsiae were enumerated after staining with the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Carlsbad, CA, USA) in a Petroff–Hausser bacterial counting chamber (Hausser Scientific, Horsham, PA, USA) and examined with a Leica microscope (Leica Microsystems, Buffalo Grove, IL, USA) (Kurtti et al. 2005). The rickettsiae were resuspended in sucrose-phosphate-glutamic acid buffer (SPG) (Feng et al. 2004) to obtain the desired inoculation dose of 1×10^7 live rickettsiae/200 µL, a dose that is at the high end of the range of

total *R. parkeri* DNA found in wild-caught *Amblyomma* ticks (Monje et al. 2014) and is similar to the dose used in previous animal models of rickettsioses (Sammons et al. 1977, Feng et al. 1993, Eremeeva et al. 2003, Bechah et al. 2007, Horta et al. 2010, Grasperge et al. 2012, Grasperge et al. 2014). The same volume of uninfected Vero cell culture was prepared in SPG as above with the exception of bacterial inoculation and counting.

2.2.2. Non-human Primates

The five adult male Indian rhesus macaques (*Macaca mulatta*) used in the study were housed at the Tulane National Primate Research Center. Practices in the housing and care of nonhuman primates conformed to the regulations and standards of the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and the Guide for the Care and Use of Laboratory Animals. The Tulane National Primate Research Center (TNPRC) is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The IACUC at the TNPRC approved all animal-related protocols specific to this study, including *R. parkeri* inoculation, tick infestation and sample collection from nonhuman primates (Protocol number: P0222) and all efforts were made to minimize animal suffering. All animals received standard primate feed as well as fresh fruit and enrichment daily, and had continual access to water. Primates were housed in pairs within treatment groups prior to and after tick infestation. Single housing was required during tick infestation in order to prevent partner manipulation of jackets and tick containment devices. Single cages are $4.3ft^2$ x 30". Pairs were housed in larger cages, which at a minimum provide at least $4.3ft^2$ x 30" per animal. Animals greater than 10 kg were allocated twice this amount of space. All animals received standard enrichment tailored to the species as dictated by the Animal Welfare Act and outlined in the TNPRC Policy on Environmental Enrichment (e.g., objects to manipulate in cage, varied food supplements,

foraging and task-oriented feeding methods, interaction with caregivers and research staff). All animal procedures were overseen by TNPRC veterinarians and their staff and their welfare was monitored daily. Complete physical exams (including evaluation of the integumentary, musculoskeletal, lymphatic, gastrointestinal, cardiovascular, and respiratory systems) were performed, and rectal temperatures and weights were taken prior to each procedure. In order to alleviate animal suffering, the macaques were anesthetized for all procedures with 5-8 mg/kg Telazol intramuscularly (IM) followed by ketamine in small increments of 2-5 mg/kg IM as needed. In addition to this anesthetic protocol, all animals were pre-emptively given 0.01 mg/kg buprenorphine IM as additional analgesia for biopsies. None of the animals in this study demonstrated any deterioration in physical condition that required euthanasia during the experiment as determined by the standard TNPRC endpoint policy; therefore, the experimental endpoint for this study was 31-35 days post-*R. parkeri*/Vero cell lysate inoculation (31-35 dpi). At this point, the macaques were humanely euthanized via administration of 5-8 mg/kg Telazol IM and 0.01 mg/kg buprenorphine IM followed by an overdose with 156 mg/kg sodium pentobarbital via intracardiac injection, a method that is consistent with the recommended guidelines of the American Veterinary Medical Association. Tulane University complies with NIH policy on animal welfare, the Animal Welfare Act, and all other applicable federal, state and local laws.

2.2.3. Tick Feeding and *Rickettsia parkeri* Inoculation

The macaques were split into three groups (Figure 2.1). Two animals each were placed in the *R. parkeri*-only and the tick + *R. parkeri* groups, and one was placed in the tick-only group. All animals were shaved and fitted with primate jackets (Lomir Biomedical, Inc., Notre-Dame

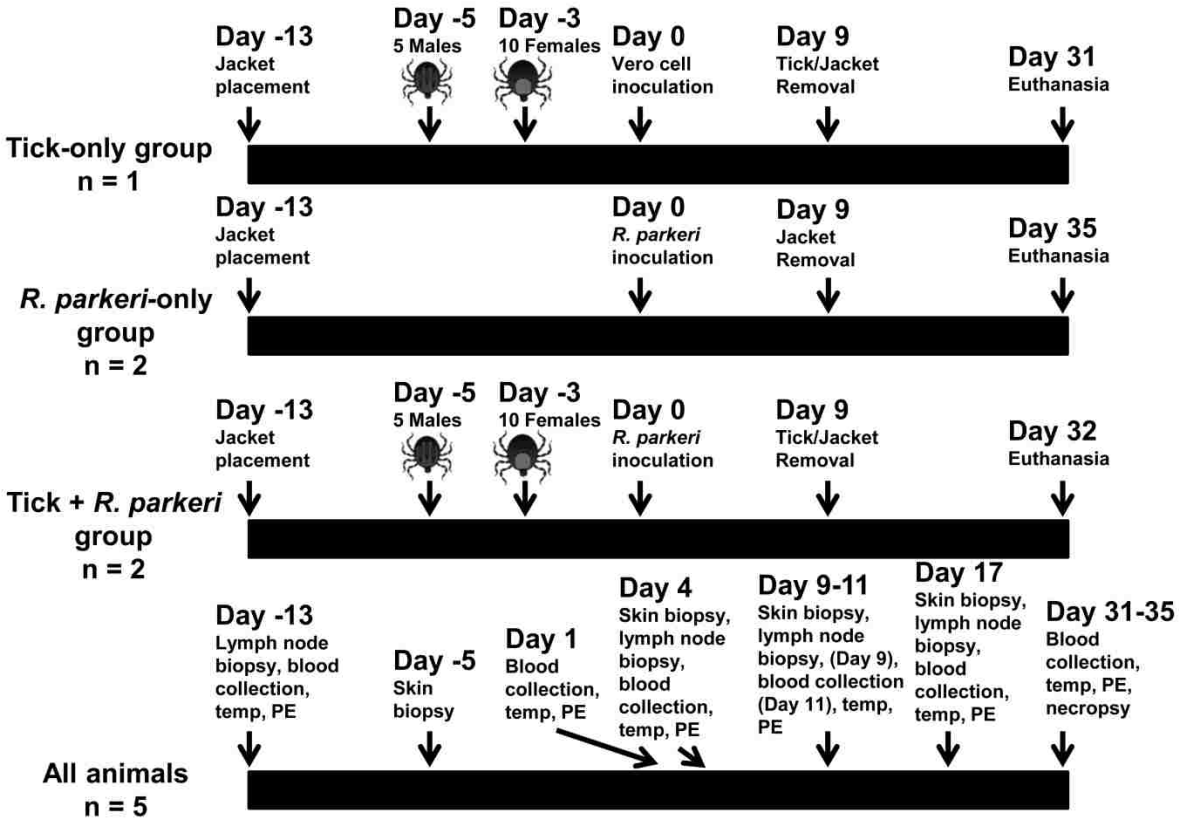

Figure 2.1. Experimental design for tick feeding, *R. parkeri*/Vero cell inoculation, and sample collection. Adult *Amblyomma maculatum* ticks were placed on the hosts as indicated. Either a partially purified low passage human isolate of *R. parkeri* or an uninfected Vero cell inoculum was administered at the indicated time points. Blood collection, physical exams (PE), rectal temperatures, and skin and lymph node biopsies were taken from all animals at the indicated time points. Complete necropsies were performed at the end of the study as indicated.

de-l'Île-Perrot, QC, Canada) one week prior to tick infestation to allow the primates to become acclimated to them. The tick exposure groups were infested with five male and ten female adult ticks using a tick containment device as previously described (Embers et al. 2013). The number of ticks was chosen based on the fact that they could comfortably feed and engorge in the space allowed within the containment device. Male ticks were placed on the host and allowed to attach one day after applying the tick containment device, followed by female tick infestation two days later to stimulate the production of pheromones secreted during male feeding, such as the

attraction-aggregation-attachment pheromone, which facilitate female tick attachment and feeding (Sonenshine 2004). The tick feeding sites and containment devices were assessed, cleaned, and reinforced as needed at 3, 7, and 12 days post female tick infestation. All of the animals were inoculated intradermally 13 days after jacket placement (3 days after female tick infestation for the tick groups) with three 200 µL injections of either partially purified Vero cell lysate or *R. parkeri* at the tick feeding site for the tick groups or at a similar location on the cranial back for the *R. parkeri*-only group. Ticks, containment devices, and jackets were removed 12 days after female tick infestation.

2.2.4. Sample Collection

For all groups, blood, skin biopsies (4-mm punch), and excisional axillary or inguinal lymph node biopsies were collected at several time points (Figure 2.1). Skin biopsies were taken both at the site of *R. parkeri* inoculation/tick feeding and away from the inoculation/infestation site on the caudal dorsum. At necropsy, skin both at the inoculation site and at a distant location from the inoculation site, axillary and inguinal lymph nodes, lung, heart, liver, spleen, and bone marrow were collected. All tissues, including biopsies and tissues collected at necropsy, were split into two portions. One portion was frozen at -20º C until DNA extraction was performed and the other portion was fixed in Z-fix fixative (Anatech, Ltd., Battle Creek, MI, USA) and routinely processed for histopathological evaluation.

2.2.5. Hematology

Blood was collected into serum separator clot tubes for serum chemistry, cytokine concentrations, and indirect enzyme-linked immunosorbent assays (ELISAs) for anti-*R. parkeri* antibody determination. Serum chemistries including aspartate aminotransferase, alanine transaminase, alkaline phosphatase, sodium, chloride, potassium, total protein, albumin,

globulin, blood urea nitrogen, creatinine, glucose, and C-reactive protein were performed immediately. Serum for cytokine evaluation and ELISAs was separated from the cellular component after centrifugation and stored at -20º C. Blood was also collected into EDTA tubes for complete blood count (CBC) determination and DNA extraction for *R. parkeri* quantification. CBCs were performed immediately, whereas blood for DNA extraction was stored at -20º C. As part of the CBC, fresh blood smears stained with Diff-Quick™ (Siemens Corporation, Washington, D.C., USA) were evaluated in a randomized manner by a board-certified veterinary clinical pathologist to determine the manual leukocyte cell differential and to evaluate erythrocyte, leukocyte, and platelet morphology.

Serum cytokine concentrations of 23 analytes (granulocyte-colony stimulating factor [G-CSF], granulocyte macrophage-colony stimulating factor (GM-CSF), interferon [IFN]-γ, IL-10, IL-12/23 (p40), IL-13, IL-15, IL-17, IL-18, IL-1 receptor antagonist [IL-1ra], IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, monocyte chemotactic protein-1 [MCP-1], macrophage inflammatory protein-1α [MIP-1α], MIP-1β, transforming growth factor-α [TGF-α], tumor necrosis factor-α [TNF-α], vascular endothelial growth factor [VEGF], soluble cluster of differentiation 40 ligand [sCD40L]) were measured with a 23 plex Milliplex MAP non-human primate cytokine magnetic bead panel (EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Each sample was evaluated in duplicate without dilution, along with duplicates of seven dilutions of provided standards and a low and high concentration quality control sample provided by the manufacturer. Data were acquired on a Luminex 100 system and analyzed using bioplex manager software (Bio-Rad Laboratories, Hercules, CA, USA).

Indirect ELISAs to detect anti-*R. parkeri* IgG were performed on the serum samples from three time points (7 days prior to *R. parkeri* exposure, 11 dpi, and 31-35 dpi) as adapted from a

previously described protocol (Graf et al. 2008). Briefly, half of the wells of 96-well plate were coated with *R. parkeri* whole cell antigen and half without antigen followed by incubation overnight at 4° C with blocking buffer (5% skim milk/0.1% Tween-20 in phosphate-buffered saline). The macaque serum samples were used as primary antibody, goat anti-monkey IgG conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) diluted 1:5000 in blocking buffer was used as the secondary antibody, and the reaction was visualized with the OptEIA™ tetramethylbenzidine substrate reagent set (BD Biosciences, San Jose, CA, USA). After a 15-minute incubation, the reaction was stopped with 2N sulfuric acid, and optical densities (ODs) were read with a Spectramax M2 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 450 nm minus the absorbance at 650 nm. Additionally, serum from a mouse previously inoculated with *R. parkeri* followed by goat anti-mouse IgG conjugated to horseradish peroxidase (Thermo Fisher Scientific, Waltham, MA, USA) as the secondary antibody and wells without serum were used as positive and negative controls, respectively. Samples were run in triplicate and the mean ODs were calculated after subtracting the ODs in the wells without antigen from the ODs in the wells with antigen. Samples that were positive at 1:64 were then subjected to two-fold serial dilutions until negative to get an endpoint titer, as has been previously reported (Paddock et al. 2008). A sample was considered positive at a certain dilution if the mean of the net ODs was greater than 0.200 or greater than the mean OD of the negative controls plus three standard deviations, whichever was larger. Endpoint titers were determined to be the highest positive dilution for each sample (Graf et al. 2008).

2.2.6. Histopathology and Immunohistochemistry

After fixation, tissues were routinely embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for histopathological evaluation. Tissue sections were evaluated in

a randomized, blinded manner by a board-certified veterinary anatomic pathologist. Skin from the inoculation sites and lymph node sections for all groups were assessed by immunohistochemistry (IHC) for the presence of *Rickettsia* using an anti-RC_{PFA} polyclonal rabbit primary antibody (Chan et al. 2011). Cross-reactivity of this antibody to *R. parkeri* was confirmed by staining *R. parkeri* (Portsmouth strain) infected Vero cells. Briefly, slides were stained using a DAKO autostainer LINK 48 after proteinase K antigen retrieval (Dako, Carpinteria, CA, USA) with anti-RC_{PFA} (1:2000) and a biotinylated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA), and visualized using the avidin/biotinylated enzyme complex (Vector Labs) and the ImmPACT™ NovaRED™ peroxidase substrate (Vector Labs), followed by counterstaining with Mayer's hematoxylin. False positives due to non-specific binding of the secondary antibody were ruled out by comparing sample staining to staining in tissue sections that were stained without primary antibody.

2.2.7. PCR for Detection of Rickettsial DNA

Genomic DNA was extracted from blood and tissue samples using the DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. Extracted DNA was stored at -80º C until real-time quantitative PCR (qPCR) was performed. In order to detect rickettsial and rhesus macaque DNA, *Rickettsia ompB* primers (Wright et al. 2011), an *R. parkeri* species-specific fluorescent-labeled probe (5'-/Cy-5/TTTG+A+G+C+A+G+CA/3IABkFQ/-3'), and rhesus macaque oncostatin M (*OSM*) primers and probe (Bruce et al. 2005) were used. The *Rickettsia ompB* gene is a single copy gene that encodes a common rickettsial surface antigen protein, and the rhesus macaque *OSM* gene is a single copy gene that encodes the oncostatin M cytokine. To quantify *R. parkeri* DNA in

macaque tissues, serial dilutions of a plasmid containing single-copies of the *R. parkeri ompB* and rhesus macaque *OSM* genes were amplified along with the unknown samples, environmental DNA extraction controls, and water (negative controls) using iTaq™ Universal Probes Supermix (Bio-Rad Laboratories) and the LightCycler® 480 system II (Roche, Indianapolis, IN, USA) as previously described (Reif et al. 2011). To confirm that the positive qPCR results were due to *R. parkeri* and to assess potential transmission of *Candidatus* "R. andeanae" (an *A. maculatum* symbiont), a 631bp segment of the *Rickettsia ompA* gene was amplified from all qPCR positive tissue sample DNA extracts and skin DNA extracts at the site of tick infestation at 4 and 9 dpi in the tick-only animal using 190.70p and 190.701 primers and thermocycling conditions as previously described (Regnery et al. 1991, Fournier et al. 1998, Pornwiroon et al. 2006). The products were visualized on a 2% agarose gel. Amplicons were extracted from the gel using a PCR Clean-up System (Promega, Madison, WI, USA), cloned into pCR 4-TOPO vector and at least five clones from each sample were sequenced at Louisiana State University. Nucleotide similarities of the sequences were evaluated on the GenBank BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Engorged female ticks from the animals in this experiment were allowed to oviposit in humidified chambers and eggs from these ticks were allowed to hatch. Genomic DNA was extracted from pools of 10-20 larvae as described above after freezing in liquid nitrogen and grinding them with a sterile pestle. Traditional PCR with the 190.70p and 190.701 primers, cloning, and sequencing were performed on the amplicons as described above.

2.3. Results

2.3.1. Tick Feeding

At 3 days post female tick placement, the majority of the female ticks had attached in all of the tick infestation groups (7/10 in the tick-only group, 9/10 and 10/10 in the tick feeding + *R. parkeri* animals). Furthermore, all males were attached at this time, except for one in the tick-only group. The remaining ticks were stuck in the glue surrounding the tick containment apparatus and did not feed. At the time of tick removal, most of the females that had attached were fully engorged in all tick infestation groups.

2.3.2. Clinical Data and Hematology

No differences in weight or temperature were noted between treatment groups during the study. Mild to marked peripheral lymphadenopathy was noted in all animals from 4 dpi to 11 dpi primarily affecting the axillary lymph nodes. At 1 dpi, moderate neutrophilia (greater than 4-fold pre-inoculation values) was noted in both primates in the tick + *R. parkeri* group as compared to mild neutrophilia (less than 3-fold baseline concentrations) in both *R. parkeri*-only primates (Figure 2.2, A). All of these animals had mild neutrophilia at 4 dpi that resolved by the time of necropsy in all animals except for macaque #1 in the tick + *R. parkeri* group. The tick-only macaque developed mild neutrophilia at 4 dpi (less than 3-fold pre-inoculation levels), with values returning to baseline at necropsy. All of the animals inoculated with *R. parkeri* were lymphopenic at 1 and 4 dpi (less than or equal to half of baseline values), except for macaque #1 in the *R. parkeri*-only group, with values returning to baseline in all animals by the date of necropsy (Figure 2.2, B). There were no apparent relevant differences between treatment groups for the rest of the CBC data.

C-reactive protein (CRP) concentration was mildly to markedly elevated (5 to greater than 50-fold increase from pre-inoculation concentrations) at 1 dpi in all *R. parkeri*-inoculated animals, with the highest concentration in primate #2 from the tick + *R. parkeri* group (Figure 2.2, C). At 4 dpi, the CRP concentrations in these animals were mildly to moderately increased (5 to 18-fold pre-inoculation values), and returned to baseline for the remainder of the study. The tick-only macaque had mild elevation (less than 4-fold) in CRP concentration at 11 dpi only.

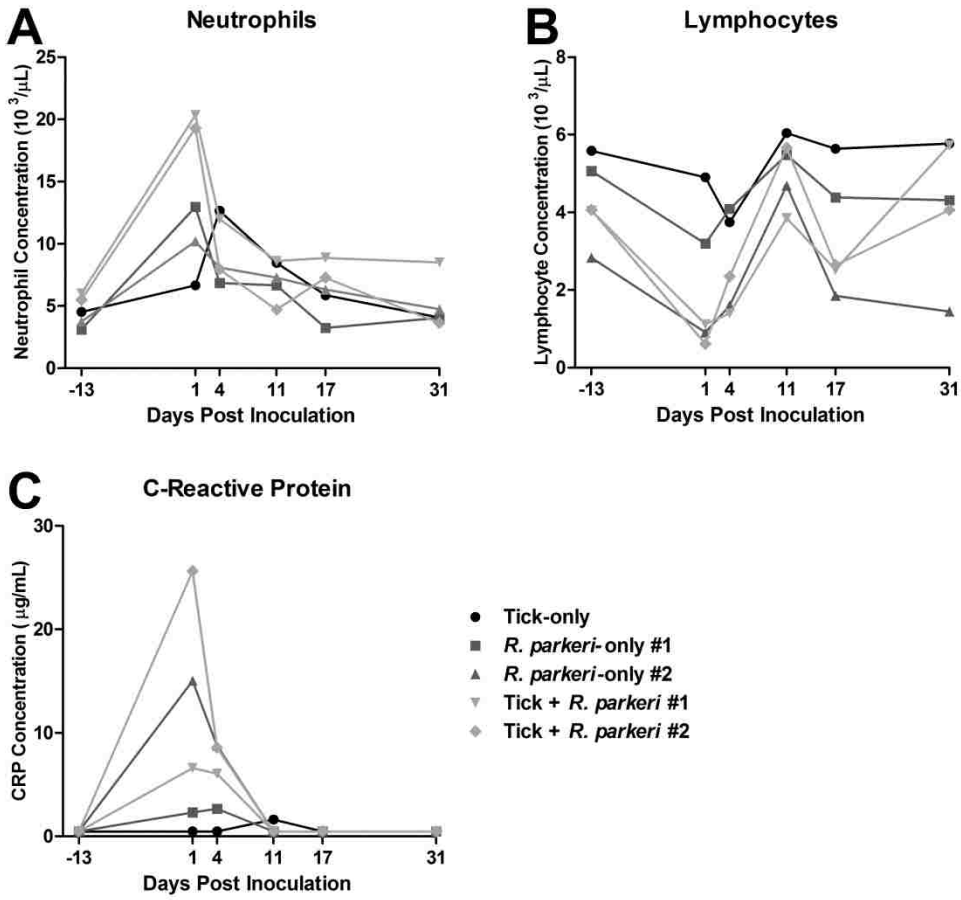

Figure 2.2. Evidence of an acute phase inflammatory response after *R. parkeri* inoculation. Comparisons of neutrophil (A), lymphocyte (B), and C-reactive protein (C) concentrations in peripheral blood of all animals at the various time points indicated. Neutrophilia, lymphopenia, and elevated C-reactive protein were noted in the acute phase of infection after *R. parkeri* inoculation with greater neutrophilia noted in the tick + *R. parkeri* group. For presentation purposes all of the final time points are plotted as 31 dpi as opposed to 31, 32, and 35 dpi for the tick-only, tick feeding + *R. parkeri*, and *R. parkeri*-only groups, respectively.

There were no apparent relevant differences between treatment groups for the rest of the chemistry analytes evaluated during the study.

There were 17-20-fold increases in IL-6 concentrations in both of the tick + *R. parkeri* macaques at 1 dpi as compared to pre-inoculation values, with moderate elevations (8-12-fold baseline concentrations) noted at 4 dpi for the same two animals and macaque #1 from the *R. parkeri*-only group (Figure 2.3, A). Moderate elevations (13-fold greater than pre-inoculation values) were noted in IFNγ concentration in primate #1 from the tick + *R. parkeri* group at 1 dpi with mild elevations (less than 7-fold pre-inoculation data) in all *R. parkeri*-inoculated animals at 4 dpi (Figure 2.3, B). Also, there were mild increases (1.7 to 2.3-fold greater than baseline) in IL-15 concentration in both animals from the tick + *R. parkeri* group as well as *R. parkeri*-only macaque #2 at 4 dpi, with mild increases (1.5-fold greater than pre-inoculation data) at 4 dpi and 11 dpi in the tick-only animal (Figure 2.3, C). There were no apparent differences between groups for the remainder of the cytokines evaluated. All animals inoculated with *R. parkeri* had anti-*R. parkeri* IgG titers of at least 1:256 at 11 dpi with at least a 4-fold increase in titers by 31-35 dpi (Table 2.1). Anti-*Rickettsia* IgG was not detected in the tick-only animal during the experiment, nor in any of the animals prior to inoculation.

2.3.3. Gross Pathology

At 4 dpi, the skin at the site of tick infestation in the tick-only animal was diffusely erythematous, raised and thickened, encompassing the majority of the 5-cm tick containment area (Figure 2.4, A). In the *R. parkeri*-inoculated animals, at 4 dpi, eschars formed at all inoculation sites (Figure 2.4, B). In the *R. parkeri*-only group, these eschars were characterized by crusted ulcers that measured approximately 0.5-1 cm in diameter and were surrounded by 0.5-1.5 cm erythematous halos. The eschars were larger in both tick + *R. parkeri* primates, with areas

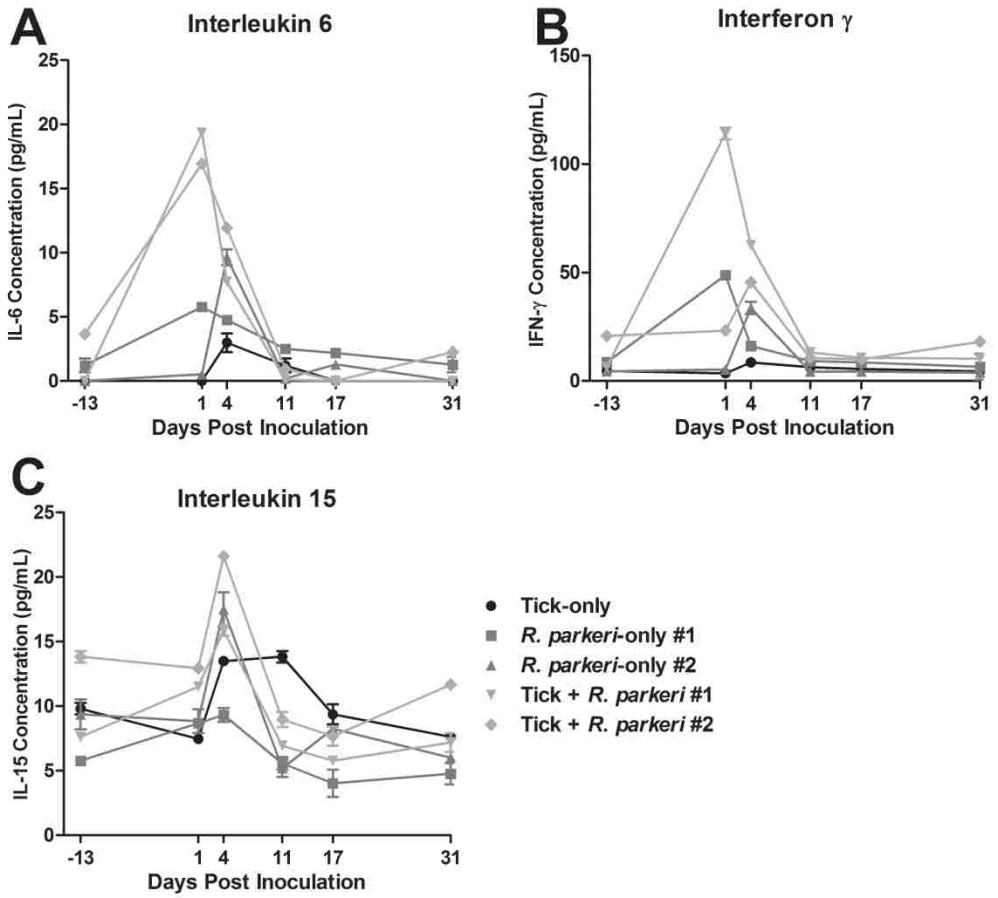

Figure 2.3. Concentrations of serum inflammatory cytokines are increased in response to *R. parkeri* inoculation. Comparisons of interleukin-6 (A), interferon γ (B), and interleukin-15 (C) concentrations in serum of all animals at the various indicated time points as determined by a magnetic cytokine bead panel kit. Measurements were performed in duplicate with the bars indicating standard error. For presentation purposes all of the final time points are plotted as 31 dpi as opposed to 31, 32, and 35 dpi for the tick-only, tick feeding + *R. parkeri*, and *R. parkeri*-only groups, respectively.

Table 2.1. Rise in anti-*R. parkeri* IgG titers in response to *R. parkeri* inoculation.

Animal	Pre-exposure (-7 dpi)	11 dpi	Necropsy (31-35 dpi)
Tick-only	-	-	-
R. parkeri-only #1	-	1:8,192	1:32,768
R. parkeri-only #2	-	1:256	1:4,096
Tick + *R. parkeri* #1	-	1:256	1:2,048
Tick + *R. parkeri* #2	-	1:256	1:4,096

All animals inoculated with *R. parkeri* had detectable anti-*R. parkeri* IgG during the acute phase of infection with at least a 4-fold increase in titers during convelscence as determined via indirect ELISA. A minus sign (-) designates that the samples are negative (titers <1:64).

of ulceration measuring up to 1.5 × 3 cm surrounded by diffusely erythematous, raised, and thickened skin of up to 5 cm in diameter (Figure 2.4, C). At 9 dpi, the *R. parkeri*-only eschars began to heal with scar formation as opposed to increased erythema and ulceration that developed in all tick infestation groups. At necropsy, eschars in the *R. parkeri*-only primates had been replaced by scars measuring up to 0.1 × 0.3 cm (Figure 2.4, D); whereas, healing ulcers with scar tissue were noted in all tick infestation groups that measured up to 1 × 2 cm in the tick + *R. parkeri* macaques (Figure 2.4, E). These healing ulcers were surrounded by maculopapular rashes measuring approximately 3-6 × 4.5-6 cm in both tick + *R. parkeri* macaques.

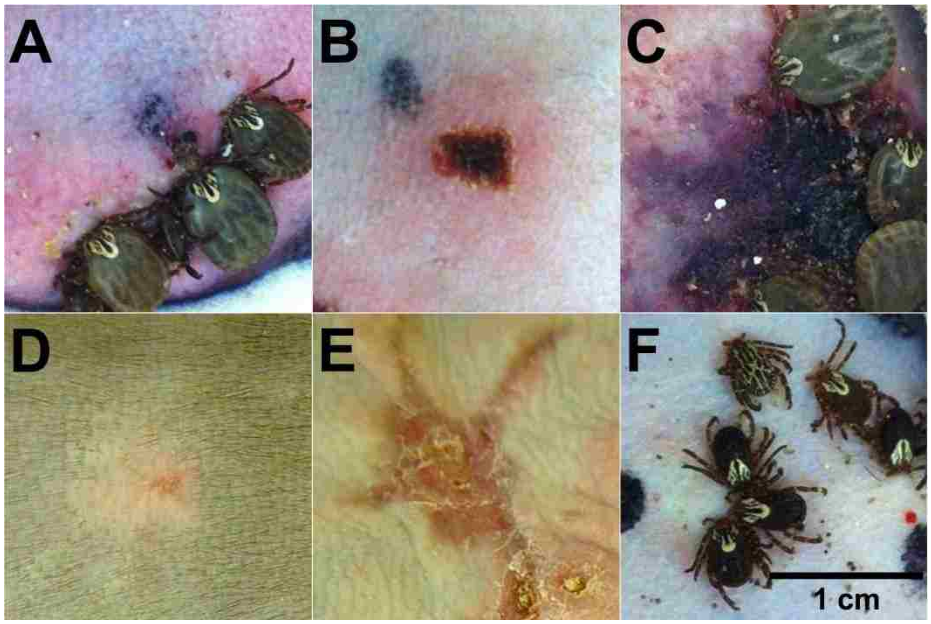

Figure 2. 4. Eschars form after intradermal *R. parkeri* inoculation and are exacerbated by tick feeding during inoculation. Photographs of gross lesions at the tick feeding/inoculation site of each group at 4 dpi (A-C), the same locations as pictured in B and C at 31-35 dpi (D and E), and another tick feeding/inoculation site at 0 dpi for comparison (F). (A) Tick feeding alone results in cutaneous erythema at 4 dpi. (B) Intradermal inoculation of *R. parkeri* results in eschar formation (well circumscribed ulcer surrounded by an erythematous halo) at 4 dpi. (C) Intradermal inoculation of *R. parkeri* during tick feeding results in a large area of necrosis surrounded by erythema at the inoculation site at 4 dpi. (D) *R. parkeri* inoculation alone results in the formation of a small scar at 35 dpi. (E) A large healing ulcer has replaced the eschar from the tick feeding + *R. parkeri* animal at 32 dpi. (F) No gross alterations are noted at the time of *R. parkeri* inoculation (3 days post female tick infestation) for comparison. Black marks were made adjacent to inoculation sites.

2.3.4. Histopathology and Immunohistochemistry

The cutaneous histologic findings are summarized in Table 2.2. At 4 dpi, marked, diffuse dermatitis extending throughout the superficial and deep dermis and characterized by infiltration of many neutrophils and fewer macrophages was observed in all macaques, except the *R. parkeri*-only macaque #1 in which the inflammation was moderate and perivascular. Epidermal necrosis was found only in the *R. parkeri*-inoculated animals (Figure 2.5, A-B, Table 2.2). At 9 dpi, moderate to marked diffuse infiltration of the superficial and deep dermis by macrophages and neutrophils was noted in the tick + *R. parkeri* animals with moderate to marked epidermal necrosis. This was opposed to mild perivascular dermatitis characterized by aggregates of variable numbers of neutrophils, macrophages, lymphocytes and plasma cells noted in the tick-only and *R. parkeri*-only macaques with mild epidermal necrosis in the *R. parkeri*-only animals. At 17 dpi and at necropsy, mild to moderate perivascular lymphocytic to lymphoplasmacytic inflammation was noted in the tick infestation groups with mild to moderate epidermal necrosis in the tick + *R. parkeri* group at 17 dpi and mild epidermal necrosis in the tick-only group at 31 dpi. The *R. parkeri*-only group had no significant histopathological lesions at these time points except for the *R. parkeri*-only macaque #1, which had mild perivascular lymphoplasmacytic inflammation at necropsy. Furthermore, marked dermal vasculitis was noted in the *R. parkeri*-only macaque #2 at 4 dpi mild vasculitis was noted in the tick + *R. parkeri* animal #1 at 4 and 9 dpi. This vasculitis was characterized by intramural fibrin deposition, endothelial cell degeneration/necrosis, and/or inflammatory cells (neutrophils and macrophages) within vessel walls (Figure 2.5, B). Mild to moderate lymphadenitis characterized by infiltrates of macrophages and neutrophils with lymphoid hyperplasia was noted in all animals at various time points after inoculation/infestation. No significant lesions were noted in the other tissues

collected. IHC revealed few to many positively staining coccobacilli primarily within macrophages and few within neutrophils in both *R. parkeri*-inoculated groups at 4 and 9 dpi (Figure 2.6, B-C, Table 2.2). Rare organisms were noted in macrophages in the tick-only animal at 4 dpi (Figure 2.6, A, Table 2.2) and in a lymph node from the tick + *R. parkeri* macaque #2 at 4 dpi.

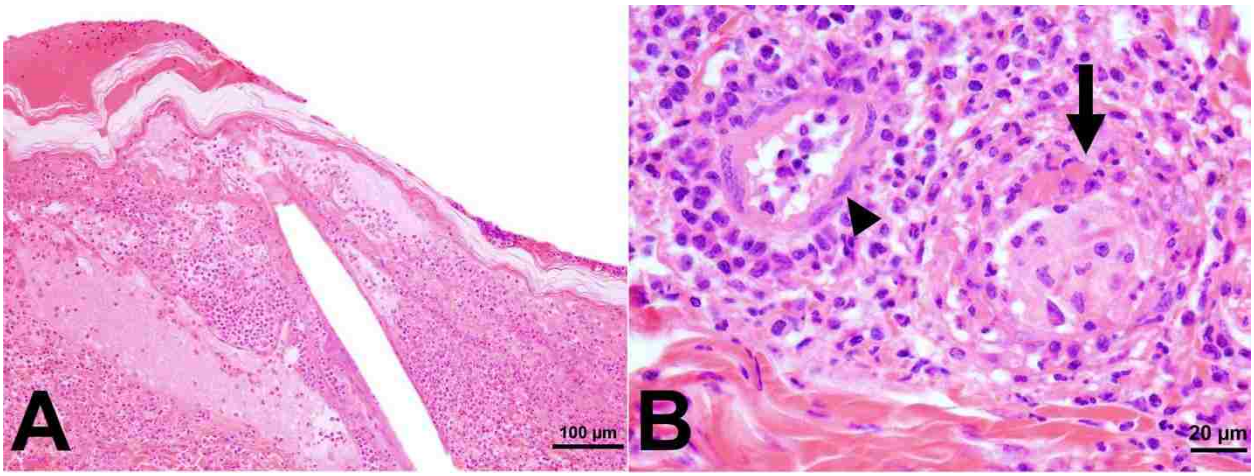

Figure 2.5. Intradermal inoculation of *R. parkeri* results in marked diffuse dermatitis. This dermatitis characterized by infiltrates of neutrophils and macrophages, epidermal necrosis, and dermal vasculitis at 4 dpi. Photomicrographs of an H&E-stained skin section from a primate from the *R. parkeri*-only group at 4 dpi. (A) The epidermis is diffusely necrotic and superficial dermis is effaced by inflammatory cells. (B) Magnified view showing a dermal vessel (arrow) effaced by neutrophils and macrophages (vasculitis) and another dermal vessel with intact endothelium (arrowhead) surrounded by neutrophils and macrophages.

Animal	Epidermal Necrosis				Dermatitis				Anti-*Rickettsia* IHC			
	4 dpi	9 dpi	17 dpi	31-35 dpi	4 dpi	9 dpi	17 dpi	31-35 dpi	4 dpi	9 dpi	17 dpi	31-35 dpi
Tick-only	0	0	0	+	+++*	+	+	++	+	0	0	0
R. parkeri-only #1	+++	+	0	0	++	+	0	+	++	++	0	0
R. parkeri-only #2	+++	+	0	0	+++*	+	0	0	+++	+++	0	0
Tick + *R. parkeri* #1	+++	+++	++	0	+++*	+++*	+	++	++	++	0	0
Tick + *R. parkeri* #2	+++	++	+	0	+++*	++*	+	++	+	++	0	0

Figure 2.6. Marked dermatitis and epidermal necrosis developed at *R. parkeri* inoculation sites. Histopathologic findings associated with intradermal inoculation of *R. parkeri* include marked epidermal necrosis and dermatitis during the acute phase of infection. Tick feeding during *R. parkeri* inoculation resulted in persistence of dermatitis in the chronic phase of infection. Anti-*Rickettsia* IHC revealed variable numbers of organisms in the skin at inoculation site during the acute phase of infection. 0 = absence of the specified parameter, + = mild histologic change (finding is rare to infrequent at high-power), ++ = moderate histologic change (change is found in multiple high-power fields or large foci are present in selected areas), +++ = marked histologic change (changes are frequently observed in multiple high-power fields or change is severe in focal areas). * Denotes diffuse dermatitis affecting the superficial and deep dermis as opposed to perivascular dermatitis denoted by the lack of an asterisk (*).

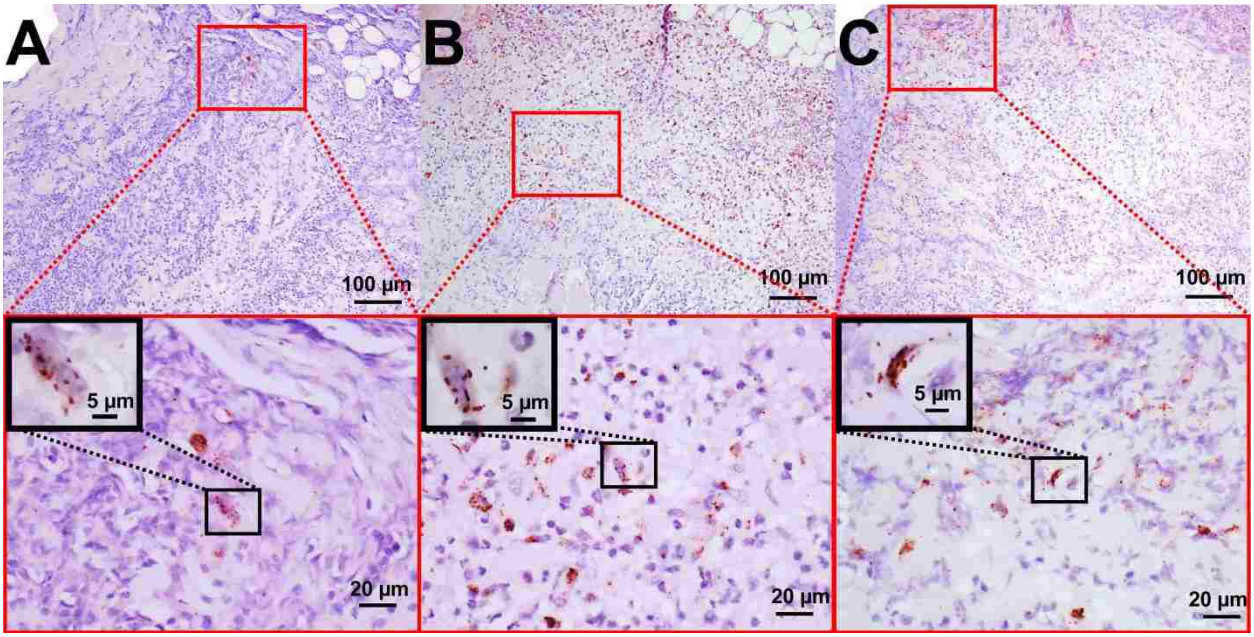

Figure 2.7. Anti-*Rickettsia* IHC demonstrating numerous organisms in the skin of animals inoculated with *R. parkeri* at 4 dpi as opposed to rare *Rickettsia* in the tick-only animal. Photomicrographs of skin sections stained with a polyclonal anti-*Rickettsia* antibody at 4 dpi. (A) Rare cells contain positive, brown-staining, rickettsial organisms in the tick-only animal. (B) Abundant positive, brown-staining, organisms in a section from an animal in the *R. parkeri*-only group. (C) Similarly, many organisms are noted in an animal from the tick + *R. parkeri* group. The red-framed images at the bottom are higher magnification views of the red-boxed areas in the top images. Black-framed insets are higher magnification images of the black-boxed areas and highlight the coccobacilli morphology of the positively stained rickettsial organisms.

2.3.5. PCR for Detection of Rickettsial DNA

R. parkeri DNA was detected in the skin at the inoculation site in all of the *R. parkeri*-inoculated animals via qPCR at 4 and 9 dpi, with lower copy numbers detected in both tick + *R. parkeri* animals at 9 dpi (Figure 2.7). Furthermore, *R. parkeri* DNA was detected at the cutaneous inoculation site at necropsy and in a lymph node at 4 dpi from the tick + *R. parkeri* monkey #2. No rickettsial DNA was detected in the other tissue or blood samples from any animal via qPCR, including all tissues from the tick-only group at all time points, the extraction and negative control samples. qPCR positive tissue samples were then subjected to traditional PCR for sequencing of a segment of rickettsial *ompA*. Sequence analysis of amplicons from all of the qPCR positive tissue samples revealed a sequence identity of ≥ 99% with several different

strains of *R. parkeri* (GenBank accession numbers CP00341.1, KF782320.1, U43802.1, FJ986616.1, JX134641.1, KC003476.1, EU715288.1, and FJ172358.1). No amplicons were observed after traditional PCR using skin DNA extracts at the site of tick infestation from the tick-only animal at 4 and 9 dpi as template. Ten of 26 (38%) engorged female ticks collected in this experiment laid eggs that produced viable larvae. Rickettsial DNA was detected in one of the 10 larval pools (10% positive). This larval pool came from a female tick from the *R. parkeri* + tick macaque #2. Sequence analysis of this amplicon revealed a sequence identity of 100% to two strains of *Candidatus* "Rickettsia andeanae" (GenBank accession numbers KF179352.1 and KF030932.1).

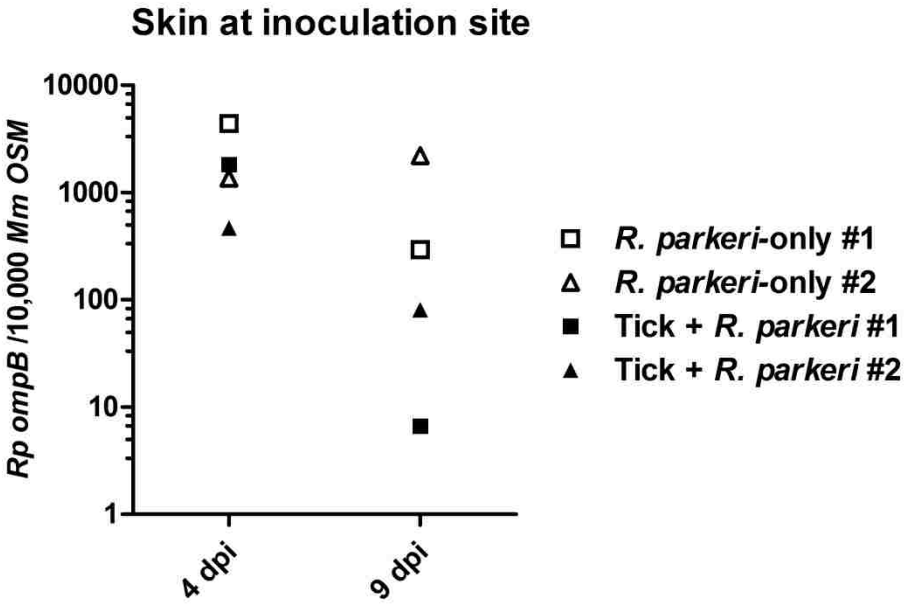

Figure 2.8. Rickettsial DNA was detected in the skin of *R. parkeri*-inoculated animals at 4 and 9 dpi. Rickettsial load as detected by qPCR in skin samples from 4 and 9 dpi expressed as *R. parkeri ompB* copies per 10,000 *M. mulatta OSM* copies. No rickettsial DNA was isolated from the tick-only macaque at any time point.

2.4. Discussion

In this study, rhesus macaques were shown to be a suitable animal model of *R. parkeri* rickettsiosis, developing an acute phase inflammatory response, lymphadenopathy, anti-*R.*

parkeri IgG, and characteristic eschars and maculopapular rashes with histologic evidence of dermal vasculitis after intradermal inoculation. The route of rickettsial inoculation used in this study, intradermal inoculation during tick feeding, while not replicating natural tick transmission of *R. parkeri*, was chosen in order to evaluate the effect of tick feeding on rickettsial pathogenesis as compared to the same dose of *R. parkeri* inoculated alone. If tick inoculation of *R. parkeri* was used instead, an appropriate *Rickettsia*-only control would be lacking as the dose and time-course of tick inoculation of *R. parkeri* remains undefined. Despite the fact that too few animals were utilized to perform statistical analysis, several conclusions can be made from this pilot study. All four rhesus macaques that were inoculated with *R. parkeri* developed an inflammatory leukogram characterized by mild to moderate neutrophilia and lymphopenia. Furthermore, moderate to marked elevations in CRP concentration, a major acute phase protein in rhesus macaques (Cray et al. 2009), and IL-6 concentration were noted during the same time frame. These abnormalities indicate activation of the innate immune response. Local inflammatory mediators, such as IL-6, are produced by innate immune cells in response to foreign substances (Murphy 2012), in this case *R. parkeri*. This leads to production of acute phase proteins, like CRP, by hepatocytes (Ceron et al. 2005, Cray et al. 2009) and release of neutrophils from the bone marrow storage pool within hours after the inciting stimulus (Stockham and Scott 2008). Inflammatory mediators also cause reduction of the circulating lymphocyte pool due to multiple factors including increased migration to inflamed tissues, increased homing to lymph nodes, and decreased migration from lymphoid tissue back to blood (Imhof and Dunon 1995). A similar pattern of inflammation was noted in experimental *R. parkeri* infection in mice (Grasperge et al. 2012) as well as in natural infection of humans with *R. conorii* (Vitale et al. 2001). Elevation of serum IFNγ and IL-15 concentrations were also

noted in *R. parkeri*-inoculated animals at 1 and 4 dpi indicating evidence of a Th1 response in these animals, which has been well described in SFG rickettsiosis (Vitale et al. 2001, Walker and Ismail 2008). Mild increases in serum IL-15 concentrations were also noted in the tick-only animal at 4 and 11 dpi, which is unexpected as tick feeding has been shown to downregulate the Th1 response (Schoeler and Wikel 2001, Brossard 2008, Kazimirova and Stibraniova 2013, Wikel 2013). While this finding could simply be an anomaly due to subject variability, further study is needed to define the role of this cytokine in the response to tick infestation. Tick feeding has also been shown to result in a Th2 response (Schoeler et al. 1999, Schoeler et al. 2000, Schoeler and Wikel 2001, Brossard 2008, Kazimirova and Stibraniova 2013, Wikel 2013); however, differences in Th2 cytokines were not detected in the serum of tick infested animals in this study, which could be attributed to the fact that these cytokines act locally at the feeding site and are not produced in large enough quantities to be detected in the peripheral blood. However, it is worth noting that many of the previous studies reporting cytokines induced by tick feeding were performed in BALB/c mice, which have a Th2-biased immune response (Locksley et al. 1987, Muller et al. 1989, Reiner and Locksley 1995). Future experiments should include evaluation of cutaneous cytokine concentrations at the tick bite site in larger numbers of non-human primates to see if the Th1 versus Th2 cytokine paradigm is valid in this species. Furthermore, all animals inoculated with *R. parkeri* developed anti-*Rickettsia* IgG titers greater than or equal to 1:256 at 11 dpi with at least a 4-fold increase in convalescent titers indicating exposure and the appropriate antibody response to the pathogen (Paddock et al. 2008). Although rickettsial infections are typically associated with fever, elevated body temperature was not detected in any of the animals during the study. All animals were anesthetized during temperature evaluations; therefore, the induced hypothermia could have masked a potential

fever. Continuous temperature monitoring could be of benefit to detect fever in future studies, as has been reported in rhesus macaques inoculated with *B. turicatae* (Lopez et al. 2014).

Experimentally-induced eschars, the hallmark gross lesions consistently found in human cases of *R. parkeri* rickettsiosis, (Paddock et al. 2004, Whitman et al. 2007, Paddock et al. 2008, Cragun et al. 2010, Romer et al. 2011, Portillo et al. 2013, Kaskas et al. 2014, Romer et al. 2014), were reproduced at all cutaneous *R. parkeri* inoculation sites in this study. Histologically these lesions were characterized by diffuse infiltrates of macrophages and neutrophils in the acute phase of infection and perivascular dermatitis with infiltrates of predominantly lymphocytes and plasma cells in the chronic phase of infection, both of which have been described in human cases of *R. parkeri* rickettsiosis (Paddock et al. 2004, Whitman et al. 2007, Paddock et al. 2008, Cragun et al. 2010, Romer et al. 2011, Kaskas et al. 2014). Furthermore, similar to human cases of *R. parkeri* rickettsiosis, dermal vasculitis was noted in two macaques inoculated with *R. parkeri* during the acute phase of infection and maculopapular rashes were noted in both macaques in the tick + *R. parkeri* group (Paddock et al. 2004, Whitman et al. 2007, Paddock et al. 2008, Cragun et al. 2010, Romer et al. 2011, Portillo et al. 2013, Romer et al. 2014). Anti-*Rickettsia* IHC confirmed the presence of organisms within cutaneous inoculation sites primarily within macrophages and occasionally within neutrophils as identified by nuclear morphology of the infected cells. The presence of *R. parkeri* primarily within inflammatory cells within cutaneous lesions as opposed to endothelial cells is similar to what is reported in the literature for human cases of *R. parkeri* rickettsiosis (Paddock et al. 2004, Whitman et al. 2007, Paddock et al. 2008, Cragun et al. 2010), an interesting finding that requires further study given the predilection of other SFG *Rickettsia* for endothelial cell infection.

Tick feeding during *R. parkeri* inoculation consistently resulted in enhanced gross lesions as well as a greater systemic inflammatory response in the acute phase of infection. Interestingly, tick feeding during *R. parkeri* inoculation did not have an effect on cutaneous rickettsial load at 4 dpi with decreased numbers of *R. parkeri* detected at 9 dpi. This is in contrast to previous studies in mice, where nymphal tick feeding post-*R. parkeri* inoculation resulted in increased bacterial load in the skin at 8 dpi (Grasperge et al. 2014). This difference could be an artifact of sampling, where, despite our best efforts, the 4-mm biopsies may not have been representative of the overall lesion. We also cannot rule out the possibility that tick feeding prior to inoculation primed the immune response leading to increased clearance of bacteria at the inoculation site. Nevertheless, *R. parkeri* DNA and rare organisms were detected in a lymph node of an animal in the tick + *R. parkeri* group by qPCR and IHC, as well as at the site of inoculation at 32 dpi by qPCR. These results suggest that tick feeding may facilitate dissemination and persistence of *R. parkeri*. However, the significance of these findings should not be overstated since they are based on data from one animal. Future study with larger animal numbers would be needed to confirm these results.

The presence of rare *Rickettsia* noted in the tick-only animal at 4 dpi in the skin by IHC is attributed to transmission of *Candidatus* "Rickettsia andeanae," a rickettsial species with no known pathogenicity, which has been detected in wild-caught *A. maculatum* (Blair et al. 2004, Jiang et al. 2005, Paddock et al. 2010, Fornadel et al. 2011, Varela-Stokes et al. 2011, Wright et al. 2011, Ferrari et al. 2012, Jiang et al. 2012, Luce-Fedrow et al. 2012, Ferrari et al. 2013, Flores-Mendoza et al. 2013, Leydet and Liang 2013, Nadolny et al. 2014, Paddock et al. 2015) and was detected in low prevalence in the larval progeny of the ticks used in this experiment. The observed transmission of low numbers of *Candidatus* "Rickettsia andeanae" has been

previously reported when persistently infected *A. maculatum* nymphs were fed on mice (Grasperge et al. 2014). Similar to this previous report, rare bacteria were noted in the tick-only animal in this study via IHC, but not by either qPCR or two rounds of traditional PCR. This finding could indicate that IHC is more sensitive than PCR in detecting low rickettsial loads after DNA extraction, or it could be a result of sampling error, where low numbers of *Rickettsia* were present in the tissue sample for IHC, but not sampled in the tissue section for PCR. The amount of disease caused by transmission of *Candidatus* "Rickettsia andeanae" in this study is uncertain, as mild peripheral neutrophilia and marked neutrophilic dermatitis were detected at 4 dpi in this animal without elevations of inflammatory cytokines or acute phase proteins in the peripheral blood. This inflammation could be attributed to the tick inoculation of bacteria; however, an inflammatory reaction to the partially purified Vero cell lysate injection or tick feeding could not be ruled out. Ideally, future study would include Vero lysate injection alone, *Candidatus* "Rickettsia andeanae" injection alone, and *Rickettsia*-free tick feeding as additional experimental groups. The lack of anti-*Rickettsia* antibody production in the tick-only animal indicates that the innate immune response alone is likely sufficient to clear the *Candidatus* "Rickettsia andeanae." Further study is needed to characterize the pathogenic potential of this organism in comparison to a known human pathogen like *R. parkeri*. However, such a study would rely upon the *in vitro* propagation of *Candidatus* "Rickettsia andeanae," which has been proven difficult to culture, growing slowly and in low numbers in mammalian, insect and tick cell lines (Luce-Fedrow et al. 2012, Ferrari et al. 2013).

In summary, rhesus macaques prove to be a valuable animal model for studying the immunobiology of *R. parkeri* rickettsiosis. Intradermal inoculation with *R. parkeri* resulted in eschar and rash formation with characteristic dermatitis, dermal vasculitis, and epidermal

necrosis that has been well described in human cases of *R. parkeri* rickettsiosis (Paddock et al. 2004, Paddock et al. 2008, Cragun et al. 2010, Kaskas et al. 2014). Tick feeding during *R. parkeri* inoculation led to increased lesion size and a greater acute phase response with increased persistence of the pathogen and inflammation in the chronic phase. Further study to characterize the influence of immunomodulatory factors introduced by tick feeding at the cutaneous interface that potentially enhance *R. parkeri* pathogenicity is required and should be considered when developing therapeutic strategies and vaccine candidates aimed at blocking transmission of SFG rickettsioses.

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CHAPTER 3
EFFECT OF *AMBLYOMMA MACULATUM* SALIVA ON THE ACUTE CUTANEOUS IMMUNE RESPONSE TO *RICKETTSIA PARKERI* INFECTION IN A MURINE MODEL

3.1. Introduction

Rickettsia parkeri rickettsiosis is a tick-borne spotted fever group (SFG) rickettsiosis characterized by fever, headache, malaise, myalgia, arthralgia, the presence of a maculopapular rash and multiple eschars (non-pruritic ulcers surrounded by erythematous halos) (Paddock et al. 2004, Whitman et al. 2007, Paddock et al. 2008, Cragun et al. 2010, Romer et al. 2011, Portillo et al. 2013, Kaskas et al. 2014, Romer et al. 2014). Since the first confirmed case of *R. parkeri* rickettsiosis in 2004 (Paddock et al. 2004), there have been at least 37 confirmed cases in the United States (Whitman et al. 2007, Paddock et al. 2008, Cragun et al. 2010, Myers et al. 2013, Ekenna et al. 2014, Kaskas et al. 2014, Paddock and Goddard 2015) in addition to several confirmed cases in South America (Romer et al. 2011, Portillo et al. 2013, Romer et al. 2014). Since the year 2000, there has been a dramatic six-fold rise in cases of SFG rickettsiosis in the United States (Groseclose et al. 2004, Adams et al. 2015). *R. parkeri* rickettsiosis has been implicated in contributing to this increase in rickettsiosis, due to suspected underreporting, which is attributable to the overlapping geographical range, clinical signs, and antibodies that cross-react with *Rickettsia rickettsii*, the agent that causes Rocky Mountain spotted fever (RMSF) (Paddock et al. 2008, Paddock 2009). Therefore, it is important to investigate the factors that contribute to the pathogenesis of this rickettsiosis, especially those that play a role in the acute phase of infection and contribute to the establishment of infection and subsequent rickettsiosis in the vertebrate host.

Once rickettsiae are inoculated by ticks into the mammalian host, they immediately come into contact with the cells and extracellular factors of the innate immune system. The cellular

infiltrate reported in biopsies of eschars from natural human cases of *R. parkeri* rickettsiosis primarily consists of macrophages and lymphocytes with rare reports of primarily neutrophilic pustules (Paddock et al. 2004, Paddock et al. 2008, Cragun et al. 2010, Kaskas et al. 2014). However, the acute phase infiltrate in natural cases is largely unknown, since most biopsies are taken several days after onset of clinical signs. In a pilot study with experimental inoculations of *R. parkeri* in rhesus macaques, neutrophils and macrophages predominated within eschars early in the course of disease with lymphoplasmacytic and histiocytic infiltrates in chronic lesions (Banajee et al. 2015). Furthermore, these animals developed an acute phase inflammatory response with elevated serum concentrations of interleukin (IL)-6 and interferon (IFN) γ (Banajee et al. 2015). Elevations of these cytokines have also been demonstrated in the serum of humans with Mediterranean spotted fever (MSF), caused by *Rickettsia conorii*, in addition to IL-10 (Vitale et al. 2001). Also, studies evaluating eschars from patients with MSF demonstrated elevated mRNA expression IFNγ, and IL-10 as compared to control skin biopsies (de Sousa et al. 2007). While these studies have characterized the acute inflammatory response induced by SFG rickettsiosis, they did not quantify the effect of factors introduced by the tick vector on this response.

As recently reviewed by Kotal et al. (Kotal et al. 2015), salivary gland extract or tick saliva from a variety of hard tick species, has been shown to alter several aspects of the innate immune system including the cellular and cytokine responses. The effects of these components on neutrophils include inhibition of granule release and reactive oxygen species, decreased chemotaxis, and inhibition of phagocytosis (Kotal et al. 2015). Similarly, phagocytosis, nitric oxide production, and cytokine production of macrophages are inhibited by tick saliva or salivary gland extract (SGE) (Kotal et al. 2015). Lastly, tick saliva has also been shown to inhibit

maturation, proliferation, and cytokine production of dendritic cells, which not only play a role in the innate immune response, but promote the development of the appropriate adaptive immune response (Kotal et al. 2015). Taken together, these effects may play a large role in how the host responds to a pathogen and the development of tick-borne diseases.

While powerful immunomodulatory effects of tick salivary components are evident based on the previously described studies, it should also be noted that the vast majority of this research is not based on immune cells found in the skin, but rather either immune cells derived from internal organs, peripheral blood, or cell lines. Furthermore, while it has been shown that tick feeding at the rickettsial inoculation site enhances rickettsial disease in murine and primate models and rickettsial proliferation in the mouse model (Grasperge et al. 2014, Banajee et al. 2015), the effect of the tick saliva on the cutaneous immune response to rickettsial infection has not been quantified. Therefore, the aim of this study was to evaluate the acute murine cutaneous immune response to *R. parkeri* with and without the influence of *A. maculatum* saliva as compared to saliva inoculation alone and untouched controls. In order to achieve these goals, cutaneous inoculation site cellular infiltrates and inflammatory cytokines were quantified at several time points within two days of each inoculation. Furthermore, inoculation site samples were evaluated via real-time quantitative PCR (qPCR) to assess for alterations in rickettsial load. We hypothesized that *A. maculatum* saliva enhances *R. parkeri* infection via downregulation of the acute cellular and cytokine immune response. The results of this study suggest that tick saliva has the ability to downregulate cellular recruitment during the acute phase of infection, but the long-term effects of this immunomodulation require further study.

3.2. Materials and Methods

3.2.1. Tick Preparation and Saliva Collection

A colony of *Rickettsia*-free *A. maculatum* were acquired from BEI resources and maintained on rodents as previously described (Troughton and Levin 2007, Grasperge et al. 2014, Banajee et al. 2015). Animal care and use for tick rearing purposes was approved by the LSU Institutional Animal Care and Use Committee (IACUC) (Protocol Number: 13-034). The ticks used in this experiment were determined to be free of *Rickettsia* via DNA extraction and traditional semi-nested PCR using the 190.70p and 190.602n and 190.70p and 190.701 primer pairs for *Rickettsia ompA* as previously described (Regnery et al. 1991, Fournier et al. 1998, Pornwiroon et al. 2006, Grasperge et al. 2014, Banajee et al. 2015). Saliva was collected from nearly fully engorged adult female ticks as previously described (Patton et al. 2012) with few modifications. These ticks were forcibly removed from adult Hartley guinea pigs (Charles River Laboratories) at approximately 7-10 days post attachment. Briefly, these ticks were taped to slides and 5 µL of 3% pilocarpine HCL (MP Biochemicals) in methanol was applied to their dorsum. A pulled 25 µL microcapillary pipet (Kimble Chase Life Science and Research Products) was applied to just their hypostome splitting the palps. The slides were then placed upright with the capillary tubes pointing down in an incubator at 37° C and saliva was collected for four hours, pooled, sterile-filtered, and stored at -80° C for further use. Prior to use, saliva protein concentration was estimated via the D_c protein assay (Bio-Rad Laboratories) according to the manufacturer's instructions. Pilocarpine concentration in the extracted saliva was determined via an electrospray ionization time-of-flight mass spectrometer 6210 (Agilent Technologies). The sample was delivered through a C8 column (Agilent Technologies) using the 1200 series high-performance liquid chromatography system (Agilent Technologies). A standard curve was

constructed using serial dilutions of a known concentration of pilocarpine (molar mass = 209 g/mol) by calculating the area under the curve for each dilution at the 209 mass to charge (m/z) peak. The concentration of pilocarpine in the saliva sample was determined by comparing the area under the curve for that sample at the 209 m/z peak to the standard curve.

3.2.2. *Rickettsia* Preparation

Partially-purified rickettsiae were recovered from *R. parkeri* (Portsmouth strain) (Paddock et al. 2004) passage 4 infected Vero cells (3 days post-inoculation) using the modified protocol of Weiss (Weiss 1973) as previously described (Petchampai et al. 2014). Rickettsiae were enumerated after staining with the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes) in a Petroff–Hausser bacterial counting chamber (Hausser Scientific) and examined with a Leica microscope (Leica Microsystems) (Kurtti et al. 2005). The rickettsiae were resuspended in sucrose-phosphate-glutamic acid buffer (SPG) (Feng et al. 2004) to obtain the desired inoculation dose of 5.5×10^6 live rickettsiae/25 µL. The same volume of uninfected Vero cell culture was prepared in SPG as above with the exception of bacterial inoculation and counting.

3.2.3. Mouse Inoculations

Animal care and use for all mouse inoculations and skin collection was approved by the LSU IACUC (Protocol Number: 13-034). Eighty male C3H/HeN mice of seven to eight weeks of age were obtained from Charles River Laboratories for use in this study (two independent experiments, forty mice per experiment). The animals were randomly divided into four experimental groups: untouched controls (n = 4/replicate), saliva only (n = 4/replicate/time point), *R. parkeri* only (n = 4/replicate/time point), and *R. parkeri* + saliva (n = 4/replicate/time point). The hair on the dorsum was clipped and the mice from the groups other than the

untouched control animals were inoculated in five spots intradermally with 25 µL of the appropriate inoculum (three cranial inoculations in line with the shoulders and two caudal in each pelvic region). For saliva inoculation alone, uninfected Vero cell lysate was prepared as previously described with addition of *A. maculatum* saliva (15 µg saliva protein per inoculation). For *R. parkeri* inoculation alone partially purified rickettsiae were prepared as described above and resuspended in SPG spiked with 16 µM pilocarpine (which equals the pilocarpine concentration that was found in each saliva inoculation). This pilocarpine was added as a control measure since this compound has been found to have inhibitory effects on lymphocyte stimulation, although this effect was noted at higher concentrations (50-500 µM) (Arzt et al. 1989, Prync et al. 1992). The last group received an inoculation of *R. parkeri* with the addition of saliva. Both rickettsiae and saliva were prepared as previously described.

3.2.4. Sample Collection

Each inoculation group was euthanized at 6 hours post inoculation (hpi), 24 hpi, or 48 hpi along with an untouched control group euthanized without any inoculation. They were then shaved and all of the hair on the dorsum was removed with a chemical depilatory agent, Nair® (Church & Dwight). The skin was then cleaned with ethanol and a 3 cm^2 area surrounding the three cranial inoculations was collected from each mouse in order to evaluate the cellular infiltrate and processed immediately as described below. Additionally two 1 cm^2 sections of skin were collected surrounding each of the caudal inoculations for cytokine concentrations and PCR evaluation of rickettsial DNA. For the untouched control animals, skin of the same dimensions was collected at similar locations on the dorsum of the animals. For cytokine analysis, the skin pieces were immediately processed as described below. Skin collected for PCR analysis was frozen at -80° C until DNA extraction could be performed.

3.2.5. Cellular Infiltrate Analysis via Flow Cytometry and Microscopy

Single cell suspensions of epidermis and dermis were made from the skin sections of each mouse for analysis of the cutaneous cellular infiltrate as previously described (Bajaña et al. 2012) with modifications. After subcutaneous fat removal, skin sections were incubated in 0.5% trypsin (Affymetrix) at 37° C for one hour and the dermis was separated from the epidermis. Epidermis and dermis were minced with dissection scissors, combined, and incubated for an additional hour at 37° C in RPMI 1640 media (Sigma Life Science) plus 5% fetal bovine serum (FBS), 3 mg/mL collagenase D (Roche Diagnostics), 1.5 mg/mL hyaluronidase (Sigma Life Sciences), and 0.2 mg/mL DNase I (Sigma Life Sciences). This suspension was then passed through a 70 μm filter and washed twice with cold Hank's balanced salt solution (Life technologies) + 5% FBS prior to staining for flow cytometry. Suspensions were stained with the following antibodies for flow cytometric analysis after blocking Fc receptors with CD16/CD32 (BD Biosciences): fluorescein isothiocyanate-labeled Ly6G, clone 1A8 (BD Biosciences), phycoerythrin labeled F4/80, clone BM8 (eBioscience), peridinin-chlorophyll protein-cyanine 5.5 labeled CD45, clone 30-F11 (BD Biosciences), and allophycocyanin labeled CD11c, clone HL3 (BD Biosciences) for 30 minutes in the dark at 4° C. The cell suspensions were then washed with 2 mL phosphate buffered saline (PBS) and fixed with 1% paraformaldehyde in PBS prior to flow cytometric analysis. All cells from each suspension were acquired using a FACSCalibur flow cytometer (BD Biosciences), and the data were analyzed using FlowJo software, version 10.1r5.

To confirm the flow cytometry findings, cytocentrifuged preparations were made from pooled skin single cell suspensions, one from each time point, for the *R. parkeri* only and *R. parkeri* + saliva groups using a Cytopro® cytocentrifuge (Wescor). These suspensions were then

stained with Diff-Quick (Siemens). The slides were then examined microscopically at 100x magnification by a board certified veterinary clinical pathologist (KHB) and percentages of neutrophils and macrophages were determined after counting 300 cells per sample.

3.2.6. Cytokine Analysis

For cytokine analysis, the skin pieces from each mouse were placed in radioimmunoprecipitation assay (RIPA) buffer with proteinase inhibitor prepared as previously described (McCracken et al. 2014) with addition of 0.2% w/v collagenase, type IV (Worthington Biochemical Corporation) and incubated at 37° C for one hour. Further digestion was achieved via a TissueLyser (QIAGEN) with glass beads as previously described (McCracken et al. 2014). The samples were centrifuged at 14,800 rpm for 20 minutes and the supernatant was stored at -80° C until cytokine analysis was performed. Cytokine concentrations of three analytes (IFN-γ, IL-6, and IL-10) were determined using a Milliplex MAP mouse cytokine magnetic bead panel (EMD Millipore) according to the manufacturer's instructions. Each sample was evaluated without dilution, along with duplicates of seven dilutions of provided standards and a low and high concentration quality control sample provided by the manufacturer. Data were acquired on a Luminex 100 system and analyzed with bioplex manager software (Bio-Rad Laboratories).

3.2.7. PCR for Detection of Rickettsial DNA

Genomic DNA was extracted from skin samples using the DNeasy Blood and Tissue Kit (QIAGEN) performed according to the manufacturer's instructions. Extracted DNA was stored at -80º C until qPCR was performed. In order to detect rickettsial and mouse DNA, *Rickettsia* 17kDa primers and an *R. parkeri* species-specific fluorescent-labeled probe were used as well as mouse *cfd* primers and fluorescent-labeled probe as previously described (Grasperge et al. 2012). The 17 kDa antigen gene encodes a common rickettsial surface antigen protein while the mouse

cfd encodes the complement factor D protein common to most mammals (Grasperge et al. 2012). To quantify *R. parkeri* DNA in mouse skin, serial dilutions of a plasmid containing single-copies of the *R. parkeri* 17kDa antigen gene and the mouse *cfd* genes were amplified along with the unknown samples, environmental DNA extraction controls, and water (negative controls) using the iTaq^{TM} Universal Probes Supermix (Bio-Rad Laboratories) and the LightCycler® 480 system II (Roche Diagnostics) as previously described (Reif et al. 2011).

3.2.8. Statistics

Data were tested for normality via the Kolmogorov-Smirnov test. Normally distributed data were expressed as means ± the standard error of the mean (SEM). Statistically significant differences of normally distributed data were determined via one-way analysis of variance (ANOVA) followed by Tukey's post hoc tests when significance was observed. Data that were not normally distributed were expressed as medians with interquartile ranges and statistically significant differences were determined via Kruskal-Wallis tests followed by Dunn's multiple comparisons when significance was observed. All statistical analyses were performed using GraphPad Prism Software version 6, and differences were considered significant at $P < 0.05$.

3.3. Results

3.3.1. *R. parkeri* Induces Cutaneous Infiltration of Macrophages and Neutrophils, Which is Inhibited by *A. maculatum* Saliva

No gross abnormalities were noted in any mice at any time point during the study, similar to what was previously reported for *R. parkeri* inoculation of C3H/HeN mice (Grasperge et al. 2012). Flow cytometry was used to determine the numbers of cutaneous neutrophils and macrophages for each experimental group after their respective inoculation as compared to untouched control mice. Neutrophils were defined as F4/80-, CD45+, Ly6G+ cells and were enumerated via the gating strategy depicted in Figure 3.1, A. Macrophages were defined as

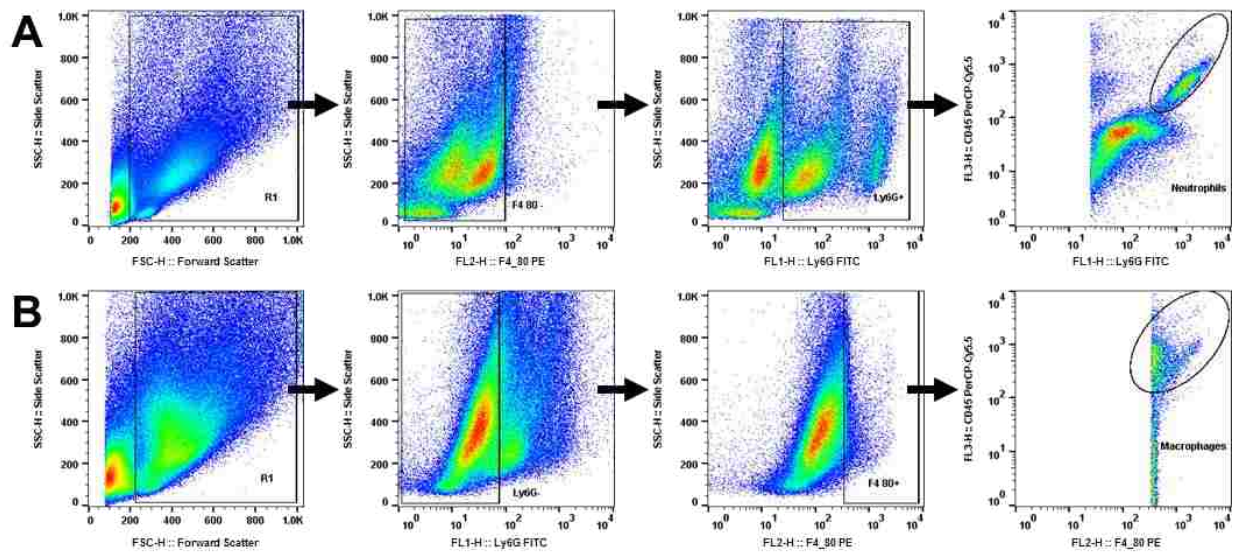

Figure 3.1. Gating strategy to determine numbers of macrophages in skin suspensions via flow cytometry. (A) Neutrophils were identified based on first eliminating cell debris followed by elimination of F4/80+ cells and then gating on cells that stained positive for Ly6G and CD45. Data shown is from the *R. parkeri* only group at 6 hpi. (B) Macrophages were identified based on first eliminating cell debris followed by elimination of Ly6G+ cells and then gating on cells that stained positive for F4/80 and CD45. Data shown is from the *R. parkeri* only group at 24 hpi.

Ly6G-, CD45+, F4/80+ cells and enumerated via the gating strategy depicted in Figure 3.1, B. A definitive population of dendritic cells could not be established via gating on a population of F4/80-, CD45+, and CD11c+ cells; therefore, they were not quantified in this study. Absolute numbers of neutrophils were significantly increased at 6 hpi for both *R. parkeri* inoculation groups as compared to the saliva only group at this time point and the untouched control group (Figure 3.2, A). Additionally at 6 hpi, neutrophil numbers were significantly decreased for the *R. parkeri* + saliva group as compared *R. parkeri* inoculation alone. Absolute numbers of macrophages were also significantly increased with respect to untouched controls for the *R. parkeri* only group at 24 and 48 hpi, as well as the saliva only and *R. parkeri* + saliva groups at 48 hpi (Figure 3.2, B). Also, there were significantly higher numbers of macrophages in the *R.*

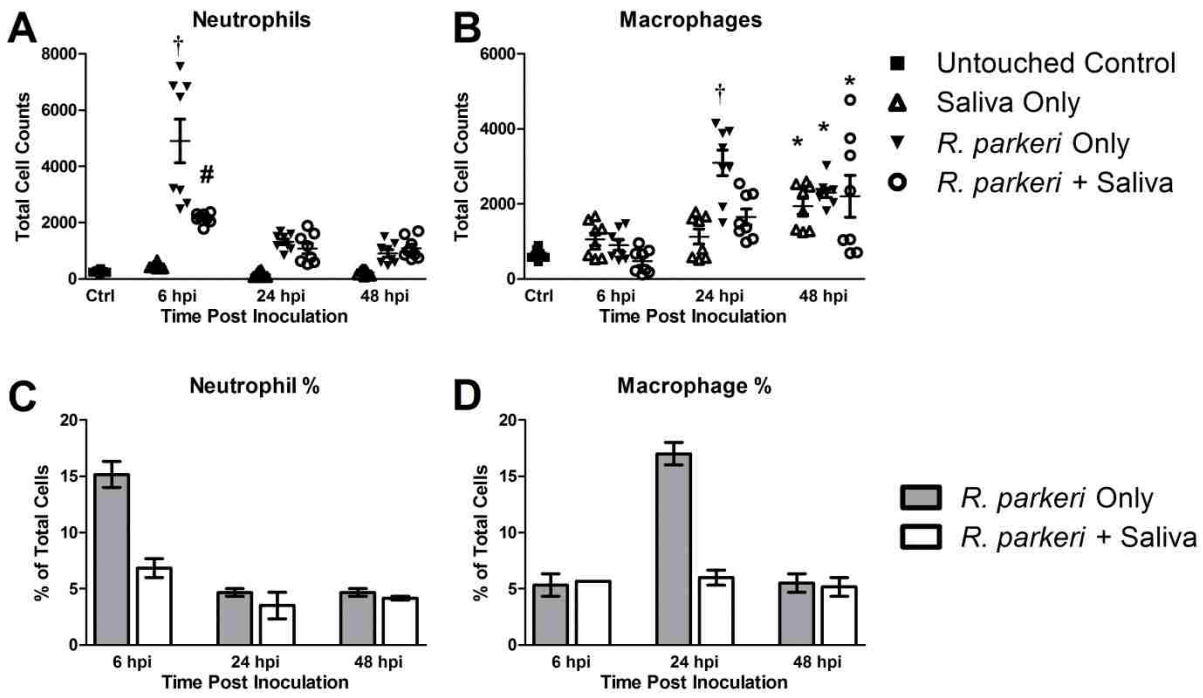

Figure 3.2. Intradermal inoculation of *R. parkeri* results in an influx of neutrophils and macrophages, which is inhibited by *A. maculatum* saliva as identified by flow cytometry and microscopy. (A, B) Flow cytometric analysis of inoculation site skin suspensions revealed significant neutrophil and macrophage influx 6 and 24 hpi of *R. parkeri*, respectively, as compared to saliva inoculation alone and untouched controls. These infiltrates were significantly inhibited by the addition of *A. maculatum* saliva to the inoculum at these time points. The data are presented as the means ± SEM. $P < 0.05$ was significant. An asterisk (*) denotes a significant difference when compared to the untouched control group, a pound sign (#) denotes a significant differences from the untouched control group and the saliva only group at the indicated time point, and a dagger (†) denotes significant differences from the untouched control group, the saliva only group, and the *Rickettsia* + saliva group at the indicated time points. Results are from two independent experiments (n = 4 mice per time point per experiment). (C, D) Microscopic evaluation of cytocentrifuged samples of inoculation site cell suspensions confirmed the flow cytometry findings that more neutrophils and macrophages were found in the inoculation sites of *R. parkeri* only animals at 6 and 24 hpi respectively when compared to inoculation of *R. parkeri* + saliva. Data are representative of pools of cell suspensions from all mice at a given time point and are from two independent experiments. Percentages of neutrophils and macrophages are taken out of total cells after counting 300 cells from each sample. The data are presented as the medians and interquartile ranges.

parkeri only group as compared to saliva only and *R. parkeri* + saliva groups at 24 hpi. In order to confirm the flow cytometry findings for the *R. parkeri*-inoculated groups, skin suspensions from each mouse within each group and time point were pooled and evaluated microscopically

after cytocentrifugation. There was a more than two-fold increase in percentage of neutrophils and macrophages at 6 hpi and 24 hpi respectively for the *R. parkeri* only group as compared to the *R. parkeri* + saliva group at these time points (Figure 3.2, C-D). Additionally, while evaluating the cytocentrifuged samples, low numbers of rickettsiae were found phagocytized mostly within macrophages and rarely within neutrophils in both *R. parkeri*- inoculated groups at all time points evaluated (Figure 3.3, A-B).

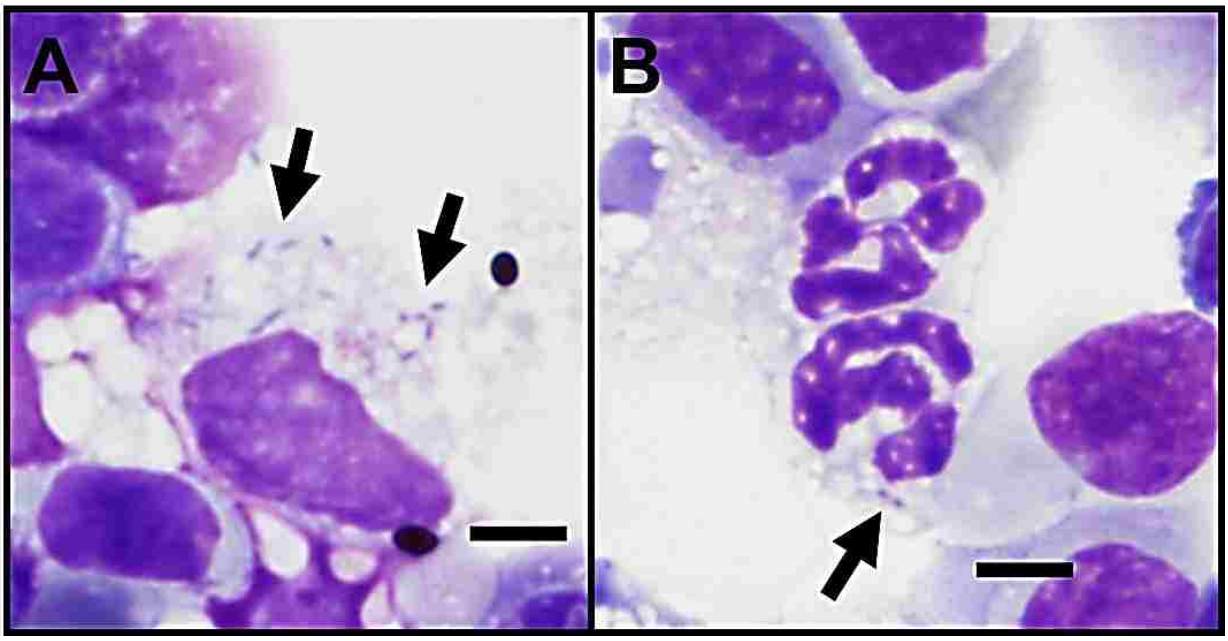

Figure 3.3. *R. parkeri* are phagocytized by macrophages and neutrophils after intradermal inoculation. Photomicrographs of cytocentrifuged preparations from mice in the *R. parkeri* only group at 6 hpi. Low numbers of *R. parkeri* (denoted by arrows) are found in macrophages (A) and neutrophils (B). Bars = 5 µm.

3.3.2. *R. parkeri* Inoculation Results in Elevated Inflammatory Cytokines, Which are Not Modulated by *A. Maculatum* Saliva

Several cutaneous cytokines that play a role in the early immune response to *Rickettsia* were evaluated at each inoculation site and in untouched controls via a cytokine magnetic bead panel. For IFNγ, *R. parkeri* inoculation alone resulted in significantly increased concentrations at 24 and 48 hpi as compared to untouched controls, with concentrations significantly increased as compared to the saliva alone group at 48 hpi (Figure 3.4, A). Additionally, *R. parkeri* + saliva

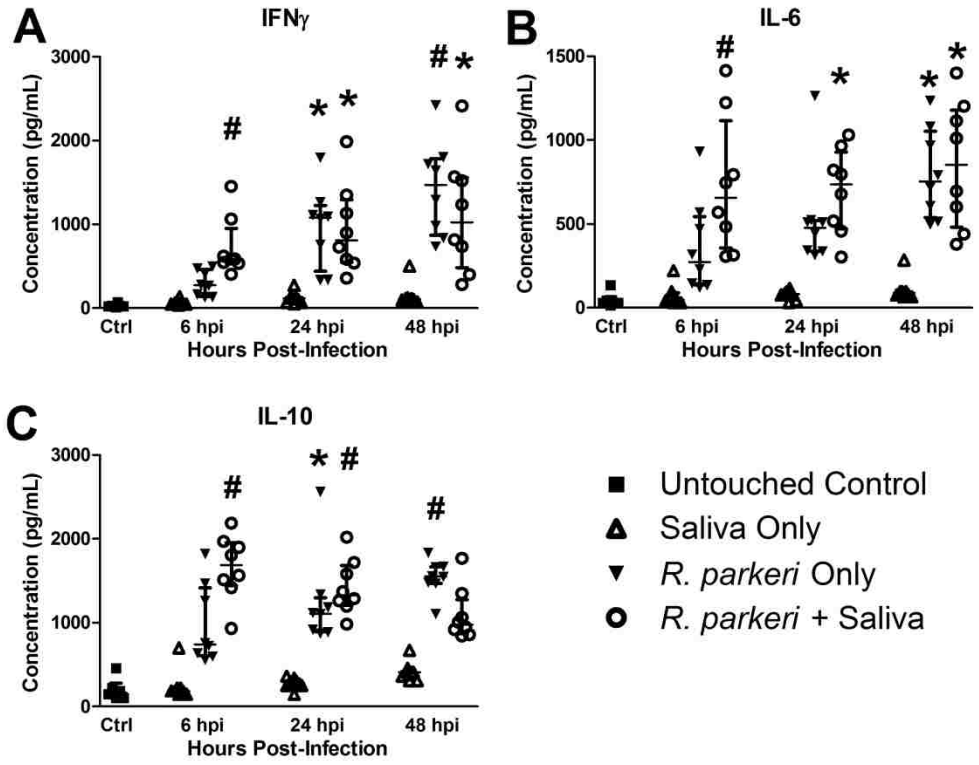

Figure 3.4. Concentrations of skin inoculation site inflammatory cytokines are increased in response to intradermal *R. parkeri* inoculation, but not significantly altered by the addition of *A. maculatum* saliva to the rickettsial inoculum. Concentrations of interferon γ (A), interleukin-6 (B), and interleukin 10 (C) at the cutaneous inoculation site were determined at the indicated time points post inoculation by a magnetic cytokine bead panel kit. These cytokines were significantly elevated at various time points both in response to *R. parkeri* inoculation alone as well as in response to *R. parkeri* inoculation with *A. maculatum* saliva. However, no significant differences were found between the *R. parkeri* only group and the *R. parkeri* + saliva group at any time point. The data are presented as the medians and interquartile ranges. $P < 0.05$ was significant. An asterisk (*) denotes a significant difference when compared to the untouched control group, and a pound sign (#) denotes a significant differences between the untouched control group and the saliva only group at the indicated time points. Results are from two independent experiments (n = 4 mice per time point per experiment).

inoculation resulted in significantly increased IFNγ concentrations at all time points as compared to untouched controls and to the saliva only group at 6 hpi. IL-6 was significantly elevated at all time points for the *R. parkeri* + saliva group as well as at 24 and 48 hpi for the *R. parkeri* only group as compared to untouched controls (Figure 3.4, B). At 6 hpi, there was also a significant

increase in IL-6 concentrations of the *R. parkeri* + saliva group as compared to the respective saliva only group at this time point. Lastly, at 24 and 48 hpi, *R. parkeri* inoculation alone resulted in significantly elevated IL-10 concentrations as compared to the untouched control group in addition to the saliva only group at 48 hpi (Figure 3.4, C). Furthermore, at 6 hpi and 24 hpi, there were significantly increased IL-10 concentrations in the *R. parkeri* + saliva group as compared to untouched controls and the saliva only groups at these time points. There were no significant differences in cutaneous cytokine concentrations between the two *R. parkeri* inoculation groups for any cytokine at any time point assessed. Also, there were no significant differences between the saliva alone group and untouched controls at any time point assessed.

3.3.3. *A. maculatum* Saliva Does Not Alter *R. parkeri* Inoculation Site DNA in the Acute Phase of Infection

To assess if there was a difference in rickettsial proliferation or destruction at the cutaneous inoculation site for *R. parkeri* inoculation only as compared to inoculation of *R. parkeri* + saliva, rickettsial load was evaluated via qPCR. Rickettsial DNA was detected at all time points assayed from the *R. parkeri* inoculation groups, but not from the untouched control or saliva only groups. There were no significant differences in rickettsial DNA between the *R. parkeri* only groups and the *R. parkeri* + saliva groups at any of the time points assessed (Figure 3.5).

3.4. Discussion

In this study, the cutaneous acute phase immune response to intradermal inoculation of *R. parkeri* was evaluated in C3H/HeN mice and compared to rickettsial inoculation with *A. maculatum* saliva as well as saliva alone and untouched controls. Flow cytometry and microscopic evaluation of single cell suspensions created from the inoculation sites demonstrated

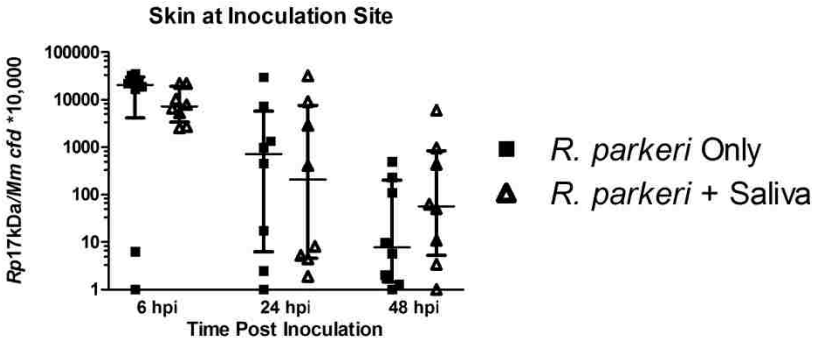

Figure 3.5. *A. maculatum* saliva did not significantly alter *R. parkeri* numbers in inoculation site skin in the acute phase after inoculation. Rickettsial load as detected by qPCR in skin samples at the indicated time points is presented as *R. parkeri* 17kDa copies per *Mus musculus cfd* copies times 10,000. No significant differences were detected between each inoculation group at each time point. No rickettsial DNA was isolated from the untouched control or saliva only mice at any time point. The data are presented as the medians and interquartile ranges. $P < 0.05$ was significant.

that *R. parkeri* inoculation resulted in an inflammatory response that was characterized by predominately neutrophils at 6 hpi and by macrophages at 24 hpi. These tissue inflammatory cell numbers were significantly higher than the saliva only and untouched control groups. A similar dermal infiltrate of neutrophils and macrophages has been reported in histologic sections of experimentally induced eschars in rhesus macaques four days after inoculation (Banajee et al. 2015). Furthermore, biopsies of human eschars caused by *R. parkeri* rickettsiosis collected later in the disease course are characterized by an influx of mononuclear cells including macrophages (Paddock et al. 2004, Paddock et al. 2008, Cragun et al. 2010, Kaskas et al. 2014). Also, similar to previous reports of natural cases of *R. parkeri* rickettsiosis in humans and experimental inoculation of SFG *Rickettsia* in experimental models, rickettsiae were microscopically detected within macrophages (Paddock et al. 2004, Whitman et al. 2007, Paddock et al. 2008, Cragun et al. 2010, Banajee et al. 2015, Riley et al. 2016). Given that this finding is in contrast to what is typically described for SFG *Rickettsia*, which have a predilection for endothelial cell infection, the role of macrophages in the progression of SFG rickettsiosis requires further study.

The current study demonstrates that *A. maculatum* saliva inhibited the cellular infiltrate induced by *R. parkeri* inoculation during the acute phase of infection. This confirms our hypothesis that *A. maculatum* saliva contains immunosuppressive properties. This hypothesis was based on the fact that *A. maculatum* saliva possesses transcripts of several anti-inflammatory molecules, such as cystatins, serpins, apyrases, and evasins (Karim et al. 2011). In fact, sialostatin L, a cystatin from another hard tick, *Ixodes scapularis*, has been shown to reduce myeloperoxidase levels in inflammatory lesions in mice which correlate to neutrophil recruitment as well as decrease the numbers of granulocytes seen histologically after *Anaplasma phagocytophilum* injection (Kotsyfakis et al. 2006, Chen et al. 2014). Likewise, evasins from *Rhipicephalus sanguineus* ticks have been found to bind to chemokines like CXCL8 (the chemokine responsible for neutrophil recruitment) and inhibit neutrophilic infiltrates in response to various stimuli (Deruaz et al. 2008). Furthermore, SGE from a variety of hard ticks, including another *Amblyomma* sp., *A. variegatum*, have been shown to inhibit the activity of CXCL8 *in vitro* (Hajnicka et al. 2001, Hajnicka et al. 2005, Vancova et al. 2007). Additionally, *Dermacentor variabilis* saliva has also been shown to inhibit murine macrophage migration *in vitro* (Poole et al. 2013). Therefore, it is not surprising that *A. maculatum* saliva was shown to inhibit cutaneous inflammation in response to *R. parkeri* inoculation potentially due to alterations in chemokines induced by saliva at the inoculation site. One of the limitations of this study is that the panel of markers used in this study (CD45, CD11c, F4/80, Ly6G) was not able to definitively identify a population of dendritic cells in mouse skin suspensions despite the fact that previous studies have used CD45 and CD11c double staining to identify these cells in the murine dermis (Dupasquier et al. 2004). It is possible that the enzymes used to prepare the skin suspensions may have cleaved CD11c off of the dendritic cells. Therefore, future studies of

cutaneous dendritic cells via flow cytometry should either be performed with different markers, such as Langerin or DC-sign or utilize a different skin disruption protocol.

Our results also indicate that *R. parkeri* inoculation both with and without saliva resulted in significant elevations of the cutaneous cytokine concentrations of IFNγ, IL-6, and IL-10 when compared to both saliva alone and untouched control groups at several time points. These results are similar to what is documented in the literature for SFG rickettsioses. Elevations in IFNγ and IL-6 have also been identified in the serum of macaques intradermally inoculated with *R. parkeri* during the acute phase of rickettsiosis as well as in humans with acute illness due to MSF (in addition to IL-10) (Vitale et al. 2001, Banajee et al. 2015). Furthermore, similar to our study IFNγ and IL-10 mRNA expression has also been shown to be elevated in biopsies of eschars of humans as compared to controls (de Sousa et al. 2007). In the current study, the addition of saliva to the *R. parkeri* inoculum did not significantly alter cutaneous cytokine concentrations as compared to inoculation of *R. parkeri* alone. As previously stated, the saliva, SGE, and various isolated salivary components from a variety of hard tick species have been shown to alter cytokine production and gene expression from murine and human immune cells or cell lines *in vitro* (Kotal et al. 2015). These studies either rely on artificial immune stimulants (i.e. concavalin A), bacterial components (i.e. lipopolysaccharide), or live viral or bacterial pathogens to assess for potential immunomodulation of tick salivary components on these cells (Kotal et al. 2015). They indicate that pro-inflammatory and Th1 cytokines such as IL-6 and IFNγ are significantly inhibited by tick salivary components, whereas concentrations of the anti-inflammatory and Th2 cytokine, IL-10, can either be unchanged, inhibited, or increased by tick salivary components (Kotal et al. 2015). In contrast to the results of these experiments and similar to the current study, when epidermal cells were isolated from C3H/HeN mice and exposed to *I. scapularis* SGE and

the pathogen *Borrelia afzelii*, the cytokine production of IL-6 and IL-10 was not altered when compared to *B. afzelii* exposure alone (Pechová et al. 2004). Further study of cutaneous immune cells is needed to determine if these cells behave uniquely when compared to cells isolated from other sources, with regards to production of cytokines in response to pathogens. The lack of a significant effect of tick saliva on the cytokine response in this study could also be potentially explained by the large variation in cytokine response seen with *R. parkeri* inoculation. This effect could simply be due to normal biological variation. However, sampling error cannot be ruled out, where the area sampled may not have been representative of the inflammatory response. Furthermore, the *R. parkeri* dose used in this study is a large dose that may have overpowered the anti-inflammatory effects of *A. maculatum* saliva despite the fact that it is at the high end of the range of total *R. parkeri* DNA found in wild-caught *Amblyomma* ticks (Monje et al. 2014), and is similar to the dose used in previous animal models of rickettsioses (Sammons et al. 1977, Feng et al. 1993, Eremeeva et al. 2003, Bechah et al. 2007, Horta et al. 2010, Grasperge et al. 2012, Grasperge et al. 2014, Banajee et al. 2015). Additional research is needed to determine the rickettsial load injected by naturally-infected ticks in order to better mimic the natural tick-host-pathogen relationship.

While rickettsial DNA was detected at all time points evaluated post-*R. parkeri* exposure at the inoculation site, *R. parkeri* tissue load was not significantly altered by *A. maculatum* saliva in the acute phase of infection. This result is in contrast to a previous study which documented that tick feeding plus *R. parkeri* inoculation resulted in increased rickettsial load in the late stages of infection as compared to *R. parkeri* inoculation alone (Grasperge et al. 2014). There are several potential reasons for this difference of results. In order to assess and quantify the mammalian immune response in an immunocompetent animal, the current set of experiments

were performed on C3H/HeN mice which have previously been shown to be resistant to *R. parkeri* infection (Grasperge et al. 2012), as opposed to the susceptible C3H/HeJ mice used in the previous study. C3H/HeJ mice have a mutation in Toll-like receptor 4, which is important to for dendritic cell function and the development of anti-rickettsial immunity (Hoshino et al. 1999, Jordan et al. 2008). Also, while not determined in vivo, the *in vitro* doubling time of SFG *Rickettsia* is reported to be 10-12 hours (Wisseman et al. 1976), therefore any effect of saliva on rickettsial proliferation, may not yet be evident in the early time points studied in this report. Additionally, the current study relied on a single injection of saliva and examined the response in the acute phase as opposed to examining rickettsial load after several days of tick feeding and a continuous exposure to tick saliva. Therefore, it is possible that the strong anti-rickettsial immune response incited by the resistant C3H/HeN mice was not altered enough by a single dose of saliva to allow for increased rickettsial proliferation in the acute phase of infection.

In summary, the experiments performed in this study allow for the *in vivo* evaluation of the local cutaneous murine immune response to a pathogen inoculated via the intradermal route. The results indicate that intradermal inoculation of *R. parkeri* induces an acute immune response characterized by neutrophils and macrophages as well as elevations in both pro-inflammatory and anti-inflammatory cytokines in the C3H/HeN mouse model. *A. maculatum* saliva, while suppressing the cellular influx does not significantly alter concentrations of these cytokines or rickettsial load in the acute phase of infection. Further study should be performed to determine if this early decrease in cellular recruitment caused by tick immunomodulation may have an impact on shaping the adaptive immune response thus enhancing rickettsial pathogenicity in the chronic phase of infection. Future studies of tick immunomodulatory factors and their interaction with

rickettsiae at the cutaneous bite site are needed in order to identify novel vaccine targets to prevent transmission of these pathogens and the subsequent development of rickettsioses.

3.5. Acknowledgements

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CHAPTER 4
DISCUSSION OF RESULTS AND FUTURE DIRECTIONS

4.1. Discussion of Results and Future Directions

Rickettsia parkeri is the cause of an emerging rickettsiosis transmitted by the Gulf Coast tick (*Amblyomma maculatum*) in the United States. Since the first description of this organism as a human pathogen in 2004 (Paddock et al. 2004), cases of *R. parkeri* rickettsiosis have now been identified in nine US states across the range of the Gulf Coast tick (Paddock et al. 2008, Cragun et al. 2010, Myers et al. 2013, Ekenna et al. 2014, Kaskas et al. 2014, Paddock and Goddard 2015). Despite this recent emergence of disease, there is a dearth of research regarding the immunology and pathogenesis of this rickettsiosis. While there is an established animal model for the recapitulation of the pathology seen with *R. parkeri* rickettsiosis in C3H/HeJ mice (Grasperge et al. 2012), these mice have a mutation in Toll-like receptor 4 and a deficient anti-rickettsial immune response (Hoshino et al. 1999, Jordan et al. 2008). Therefore, two other immunocompetent mammals were used in the present studies, rhesus macaques and C3H/HeN mice. These newly established models can be used in future rickettsial immunology research, because they produce a fully functional immune response and may better mimic the disease found in humans.

There is also a need for studies that explore the various factors that lead to establishment of *R. parkeri* infection in the mammalian host including the role of tick-associated molecules that are inoculated into the cutaneous bite site along with rickettsiae. Based on research on other hard tick species, we know that tick saliva, salivary gland extract (SGE), or specific salivary molecules have immunosuppressive properties on a variety of immune cell types (Kotal et al. 2015). However, these experiments, designed to characterize immunomodulatory attributes, were mostly performed *in vitro* or on immune cells derived from internal organs rather than the

cutaneous inoculation site. Consequently, the studies presented in this dissertation fill a need by addressing the effect of tick immunomodulation on rickettsial immunity at the cutaneous inoculation site. The overall hypothesis is that if tick saliva has immunomodulatory capabilities, then these factors enhance rickettsial infection via downregulation of the host immune response.

To address the need for an immunocompetent animal model for *R. parkeri* rickettsiosis that would effectively replicate natural disease and explore the role of tick feeding in pathogenesis, rhesus macaques were utilized. These animals were intradermally inoculated with *R. parkeri* both alone and during adult *A. maculatum* feeding and the disease in these animals was compared to an animal infested with adult ticks alone. In order to asses both rickettsial load and immune response to infection, peripheral blood, lymph nodes, and skin at the inoculation sites were evaluated at several time points during the study. Using this system, we were able to test our hypothesis that by modulating the host immune response, tick feeding enhances infection and pathology of spotted fever group (SFG) *Rickettsia* in the mammalian host.

As opposed to the tick-only animal, all *R. parkeri*-inoculated macaques developed inflammatory leukograms characterized by neutrophilia and lymphopenia, as well as elevated C-reactive protein and interleukin (IL)-6 concentrations (acute phase inflammatory proteins) post-inoculation of *R. parkeri*. This acute phase inflammatory response is similar to what has previously been reported in an experimental mouse model of *R. parkeri* rickettsiosis (Grasperge et al. 2012) and human cases of Mediterranean spotted fever (Vitale et al. 2001). Elevated serum Th1 cytokines (interferon [IFN]γ, IL-15) were also detected in the acute phase after inoculation of *R. parkeri*, which has also been described in SFG rickettsiosis (Mansueto et al. 1994, Vitale et al. 2001, Walker and Ismail 2008). Additionally, cutaneous eschars, the characteristic lesions reported in human *R. parkeri* rickettsiosis (Paddock et al. 2004, Whitman et al. 2007, Paddock et

al. 2008, Cragun et al. 2010, Romer et al. 2011, Portillo et al. 2013, Kaskas et al. 2014, Romer et al. 2014) formed at all *R. parkeri* inoculation sites, as opposed the tick infestation control animal. The eschars were characterized grossly by cutaneous ulceration surrounded by erythema. Histologically, the dermis of these animals was infiltrated by numerous macrophages and neutrophils and had evidence of vasculitis. These results indicate that rhesus macaques are a good model of *R. parkeri* rickettsiosis, which develop the characteristic pathological manifestations of disease after cutaneous inoculation.

In addition to the inflammatory response described above, animals inoculated with *R. parkeri* during tick feeding had greater neutrophilia and IL-6 concentrations as compared to the *R. parkeri* only groups. Furthermore, larger and slower healing eschars were observed in the tick feeding plus *R. parkeri* group as compared to the group inoculated with *R. parkeri* alone. Also, enhanced dissemination of *R. parkeri* to draining lymph nodes early in infection and increased persistence at the inoculation site were observed in the tick plus *R. parkeri* group. These results indicate that tick feeding enhanced the disease induced by intradermal *R. parkeri* inoculation. Despite the interesting conclusions that were derived from this study, there are several limitations of this research that should be addressed in future studies. First, the number of animals in the current study was too low to perform statistical analysis. Therefore, future studies should include larger numbers of primates if financially feasible. Also, the animals in this report did not develop a fever, which is uniformly reported in human patients with *R. parkeri* rickettsiosis (Paddock and Goddard 2015). This effect is likely due to the fact that the animals were anesthetized during temperature recording, therefore the induced hypothermia could have masked a fever. Future studies may utilize telemetry to continuously monitor temperature in these animals and detect a fever. Additionally, elevated Th2 cytokines were not detected in any

of the primates in the tick feeding groups, which has been reported in other studies evaluating the immune response to tick feeding or salivary compounds (Kotal et al. 2015). The possibility that these cytokines may have been elevated locally at the bite site but not systemically in the serum is a hypothesis that requires further investigation. Lastly, rare rickettsial organisms were found via immunohistochemistry within dermal macrophages at the cutaneous bite site in the tick only group. This is likely due to the fact that these ticks were infected with *Candidatus* "Rickettsia andeanae". Interestingly, this group did not develop cutaneous eschars in response to this inoculation. One hypothesis for this difference in disease is that ticks infected with the non-pathogenic rickettsial species release low numbers of *Rickettsia* into the bite site when feeding as opposed to the larger numbers inoculated by ticks infected with *R. parkeri*. Another possibility is that there may be inherent differences in pathogenicity in these organisms due to various undetermined virulence factors. Future research is required in order to identify why there is a difference in pathogenicity for various rickettsial species, and should include study of the tick-related factors that may play a role in altered virulence. Despite these limitations, the results of this study suggest that immunomodulatory factors introduced during tick feeding may enhance the pathogenicity of SFG *Rickettsia*, and their role in the establishment of rickettsial infection at the cutaneous inoculation site requires further investigation.

While the previously described work provides evidence of acute inflammation in *R. parkeri* rickettsiosis and an effect of tick feeding augmenting this acute inflammatory response, this immunomodulation was not quantified and fully evaluated at the cutaneous inoculation site. Therefore, further study was performed in C3H/HeN mice to characterize the immune response to intradermal inoculation of *R. parkeri* during the acute phase of infection in the presence and absence of tick saliva as compared to saliva inoculation alone and untouched controls. The

hypothesis was that by downregulating the acute innate immune response, *A. maculatum* saliva enhances *R. parkeri* infection in the mammalian host. Flow cytometric analysis of cutaneous inoculation site cell suspensions showed that there was a significant increase in the cellular influx of neutrophils and macrophages at 6 and 24 hours post inoculation, respectively, as compared to saliva inoculation alone and untouched controls, similar to what was reported in the primate study. This infiltrate was significantly downregulated by the addition of *A. maculatum* saliva to the inoculum at these time points, which is consistent with the anti-inflammatory effect of tick saliva described in the literature (Hajnicka et al. 2001, Hajnicka et al. 2005, Kotsyfakis et al. 2006, Vancova et al. 2007, Deruaz et al. 2008, Chen et al. 2014).

Furthermore, three cytokines were evaluated at all cutaneous inoculation sites and in untouched controls: IFNγ, IL-6, and IL-10. All three of these cytokines were significantly increased in response to *R. parkeri* inoculation at various time points both with and without the addition of *A. maculatum* saliva as compared to saliva inoculation alone and/or untouched controls. This cytokine response to SFG rickettsiosis is consistent with what is reported in the literature for SFG rickettsiosis (Mansueto et al. 1994, Vitale et al. 2001, de Sousa et al. 2007) and in the previously presented primate model of *R. parkeri* rickettsiosis. However, there were no differences in cytokine concentrations between the *R. parkeri* only and *R. parkeri* + saliva groups at any time point evaluated, unlike what has been reported with *in vitro* tick saliva studies (Kotal et al. 2015). This discrepancy may be due to the fact that cutaneous immune cell responses may differ from those reported in other *ex vivo* studies, where cells were isolated from internal organs. However, the cytokine concentrations in *R. parkeri*-inoculated animals were highly variable, possibly due to a sampling error, where samples that were fully representative of the inoculation site may not have been collected. Lastly, rickettsial tissue load as evaluated by

quantitative real-time PCR was not significantly affected by the addition of *A. maculatum* saliva to the *R. parkeri* inoculum at any time point evaluated. This may be due to the combination of the nature of the C3H/HeN mice that produce a strong immune response to the pathogen, and/or due to the large rickettsial dose that was administered, which may have overpowered the ant-inflammatory effects induced by saliva in the acute phase of infection. Furthermore, as the rickettsial doubling time is approximately 10-12 hours *in vitro* (Wisseman et al. 1976), an effect of saliva on rickettsial proliferation may not be apparent within 48 hours post infection. The results of this work describe the acute immune response to *R. parkeri* and how it is impacted by the saliva of its vector. They pave the way for future research into tick immunomodulatory molecules and their effect on *Rickettsia* transmission and pathology.

Despite the studies described in this dissertation laying down a solid foundation, future research of *R. parkeri*, the mammalian immune response induced by this pathogen and how that immune response is augmented by the feeding of *A. maculatum* is still needed. For example, future studies should include investigations of how tick feeding affects the cutaneous rickettsial response of other inflammatory cells such as dendritic cells or NK cells early in the infection and then how these alterations shape the adaptive cell-mediated immune response and progression of disease. Furthermore, research of tick saliva-induced immunomodulatory effects on rickettsial killing by inflammatory cells such as macrophages, dendritic cells, neutrophils, NK cells and cytotoxic T cells should also be explored. These studies could ultimately lead to the discovery of tick salivary molecules necessary for rickettsial establishment in the mammalian host and the development of potential transmission-blocking therapeutics that target these molecules.

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APPENDIX A
COMMONLY USED ABBREVIATIONS

ANOVA – analysis of variance

BLAST – basic local alignment search tool

CBC – complete blood count

CD – cluster of differentiation

cfd – complement factor D

CRP – C-reactive protein

DLAM – Division of Laboratory Animal Medicine

DNA – deoxyribonucleic acid

dpi – days post inoculation

EDTA - ethylenediaminetetraacetic acid

ELISA – enzyme-linked immunosorbent assay

FBS – fetal bovine serum

G-CSF – granulocyte colony stimulating factor

GM-CSF – granulocyte macrophage-colony stimulating factor

H&E – hematoxylin and eosin

hpi – hours post inoculation

IACUC – Institutional Animal Care and Use Committee

IFN - interferon

IgG – immunoglobulin G

IHC – immunohistochemistry

IL – interleukin

IM – intramuscularly

kDa - kiloDalton

LPS – lipopolysaccharide

LSU – Louisiana State University

MCP-1 – monocyte chemotactic protein 1

MHC – major histocompatibility complex

mRNA – messenger ribonucleic acid

MSF – Mediterranean spotted fever

NK – natural killer

NO – nitric oxide

OD – optical density

OSM – oncostatin M

PCR – polymerase chain reaction

PE – physical exam

qPCR – quantitative real-time polymerase chain reaction

RIPA – radioimmunoprecipitation assay

RMSF – Rocky Mountain spotted fever

RNA – ribonucleic acid

rOmp – rickettsial outer membrane protein

RPMI – Roswell Park Memorial Institute

Sca – surface cell antigen

sCD40L – soluble cluster of differentiation 40 ligand

SEM – standard error of the mean

SFG – spotted fever group

SGE – salivary gland extract

SPG – sucrose phosphate-glutamic acid

Th1 – type 1 helper T cell

Th2 – type 2 helper T cell

TLR – Toll-like receptor

TNF – tumor necrosis factor

TNPRC – Tulane National Primate Research Center

VEGF – vascular endothelial growth factor

APPENDIX B
CONSENT FORMS

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