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Research article

Effect of *Amblyomma maculatum* (Acari: Ixodidae) Saliva on the Acute Cutaneous Immune Response to *Rickettsia parkeri* Infection in a Murine Model

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Abstract

Rickettsia parkeri Luckman (Rickettsiales: Rickettsiaceae) is a pathogenic spotted fever group *Rickettsia* transmitted by *Amblyomma maculatum* Koch (Acari: Ixodidae) in the United States. The acute innate immune response to this pathogen and the effect of tick feeding or salivary components on this response is largely unknown. We hypothesized that *A. maculatum* saliva enhances *R. parkeri* infection via downregulation of the acute cellular and cytokine immune response. C3H/HeN mice were intradermally inoculated with *R. parkeri* both with and without *A. maculatum* saliva. Flow cytometry and microscopic evaluation of inoculation site skin suspensions revealed that neutrophils and macrophages predominated at 6 and 24 h post *R. parkeri* inoculation, respectively. This cellular influx was significantly downregulated when *A. maculatum* saliva was inoculated along with *R. parkeri*. Inflammatory cytokines (interferon γ and interleukins 6 and 10) were significantly elevated after *R. parkeri* inoculation. However, cytokine concentration and rickettsial load were not significantly modified by *A. maculatum* saliva during the acute phase of infection. These results revealed that tick saliva inhibits the cutaneous cellular influx during the acute phase of rickettsial infection. Further study is needed to determine the overall impact of this effect on the establishment of rickettsiosis in the host and development of disease.

Key words: Gulf Coast tick, host–pathogen–vector interaction, innate immunity, spotted fever group rickettsiosis

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 (nonpruritic 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 in addition to several confirmed cases in South America (Paddock and Goddard 2015). Since the year 2000, there has been a dramatic sixfold rise in cases of SFG rickettsiosis in the United States (Groseclose et al. 2004, Adams et al. 2015). *Rickettsia parkeri* rickettsiosis has been implicated in contributing to this increase in rickettsiosis. This effect is likely due to 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 (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 predominantly 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 of 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. (2015), salivary gland extract (SGE) 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 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. This time frame was chosen based on previous studies with *R. parkeri*, where the majority of the cutaneous pathology and inflammation was centered around the acute phase of infection (Banajee et al. 2015). We hypothesized that *A. maculatum* saliva enhances *R. parkeri* infection via downregulation of the acute cellular and cytokine immune response.

Materials and Methods

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 seminested 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). 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, Wilmington, MA) at ~7–10 d post attachment. Briefly, these ticks were taped to slides and 5 µl of 3% pilocarpine HCL (MP Biomedicals, Santa Ana, CA) in methanol was applied to their dorsum. A pulled 25 µl microcapillary pipet (Kimble Chase Life Science and Research Products, Vineland, NJ) 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 4 h, 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, Hercules, CA) 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, Wilmington, DE). 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.

Rickettsia Preparation

Partially purified rickettsiae were recovered from *R. parkeri* (Portsmouth strain; Paddock et al. 2004) passage 4 infected Vero cells (3 d postinoculation) using the modified protocol of 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) in a Petroff–Hausser bacterial counting chamber (Hausser Scientific) and examined with a Leica microscope (Leica Microsystems, Buffalo Grove, IL; 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. Since the amount of *R. parkeri* inoculated by ticks during natural feeding is unknown, this dose was chosen based on the fact that it is within the range reported in wild-caught ticks (Monje et al. 2014), and similar to the dose used with other animal models of SFG rickettsiosis (Feng et al. 1993, Bechah et al. 2007, Grasperge et al. 2012, Grasperge et al. 2014, Banajee et al. 2015). The same volume of uninfected Vero cell culture was prepared in SPG as above with the exception of bacterial inoculation and counting.

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 7–8 wk of age were obtained from Charles River Laboratories for use in this study (two independent experiments, 40 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.

Sample Collection

Each inoculation group was euthanized at 6 h postinoculation (hpi), 24 hpi, or 48 hpi along with an untouched control group euthanized without any inoculation. The mice were humanely euthanized with carbon dioxide followed by cervical dislocation. They were then shaved and all of the hair on the dorsum was removed with a chemical depilatory agent, Nair (Church & Dwight, Ewing, NJ). 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.

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, Santa Clara, CA) at 37°C for 1 h 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, St. Louis, MO) plus 5% fetal bovine serum (FBS), 3 mg/ml collagenase D (Roche Diagnostics, Indianapolis, IN), 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, Carlsbad, CA) + 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, San Jose, CA): fluorescein isothiocyanate-labeled Ly6G, clone 1A8 (BD Biosciences), phycoerythrin-labeled F4/80, clone BM8 (eBioscience, San Diego, CA), peridinin-chlorophyll protein-cyanine 5.5-labeled CD45, clone 30-F11 (BD Biosciences), and allophycocyanin-labeled CD11c, clone HL3 (BD Biosciences) for 30 min 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, Logan, UT). These suspensions were then stained with Diff-Quick (Siemens, Washington, D.C.). The slides were then examined microscopically at 100× magnification by a board-certified veterinary clinical pathologist (K.H.B.) and percentages of neutrophils and macrophages were determined after counting 300 cells per sample.

Cytokine Analysis

For cytokine analysis, the skin pieces from each mouse were placed in radioimmunoprecipitation assay 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 1 h. Further digestion was achieved via a TissueLyser (QIAGEN, Germantown, MD) with glass beads as previously described (McCracken et al. 2014). The samples were centrifuged at 14,800 rpm for 20 min 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, Billerica, MA) 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).

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 −80°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 Universal Probes Supermix (Bio-Rad Laboratories) and the LightCycler 480 system II (Roche Diagnostics) as previously described (Reif et al. 2011).

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 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 $P < 0.05$.

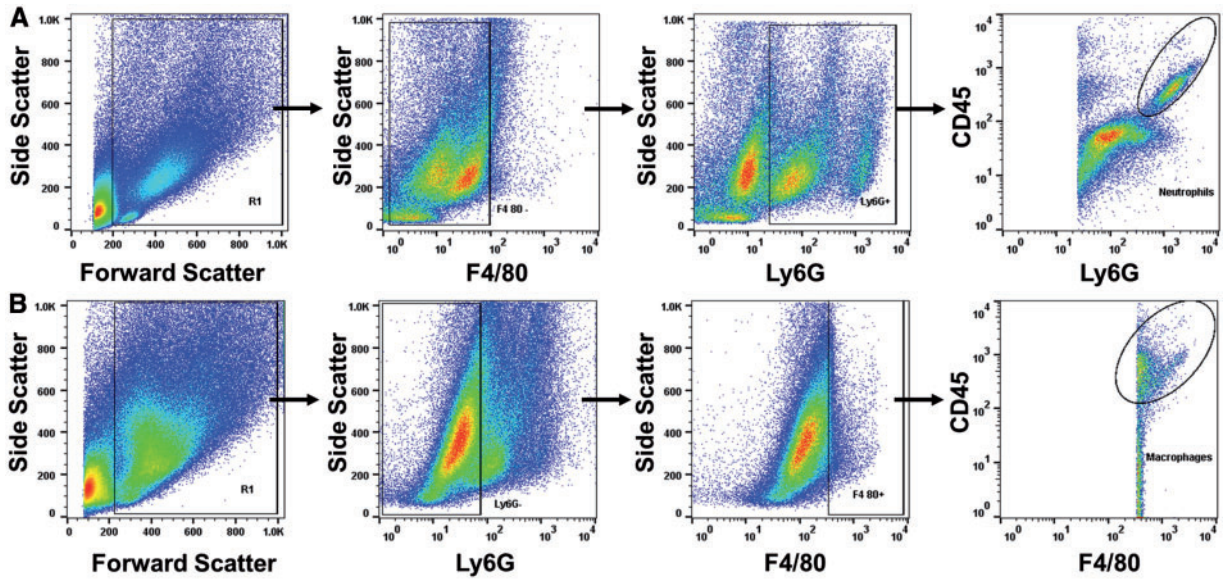

Fig. 1. Gating strategy to determine numbers of macrophages in skin suspensions via flow cytometry. (**A**) Neutrophils were identified based on first eliminating cell debris followed by elimination of F4/80+ cells and then gating on cells that stained positive for Ly6G and CD45. Data shown are from 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 are from the *R. parkeri* only group at 24 hpi.

Results

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+, and Ly6G+ cells and were enumerated via the gating strategy depicted in Fig. 1A. Macrophages were defined as Ly6G-, CD45+, and F4/80+ cells and enumerated via the gating strategy depicted in Fig. 1B. 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 (Fig. 2A). 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 (Fig. 2B). Also, there were significantly higher numbers of macrophages in the *R. 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 twofold 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 (Fig. 2C–D). Additionally, while evaluating the cytocentrifuged samples, low numbers of intact rickettsiae were found phagocytized mostly within macrophages and rarely within neutrophils in both *R. parkeri*-inoculated groups at all time points evaluated (Fig. 3).

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 (Fig. 4A). Additionally, *R. parkeri* + saliva 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 (Fig. 4B). 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 (Fig. 4C). 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.

A. maculatum Saliva does not Alter *R. parkeri* Load at the Inoculation Site 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

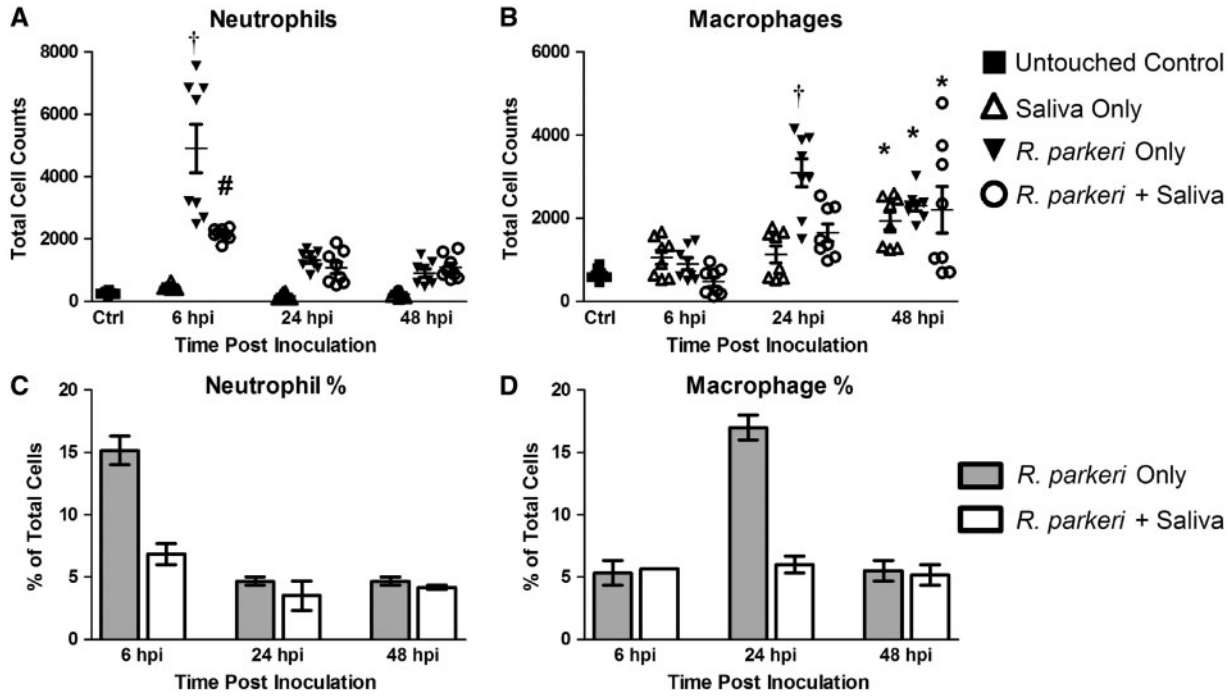

Fig. 2. Intradermal inoculation of *R. parkeri* results in an influx of neutrophils and macrophages, which is inhibited by *A. maculatum* saliva as identified by flow cytometry and microscopy. (**A**, **B**) Flow cytometric analysis of inoculation site skin suspensions revealed significant neutrophil 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 difference 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.

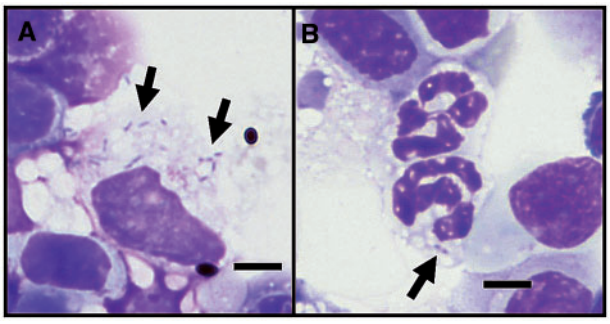

Fig. 3. *R. parkeri* are phagocytized by macrophages and neutrophils after intradermal inoculation. Photomicrographs of cytocentrifuged preparations from mice in the *R. parkeri* only group at 6 hpi. Low numbers of *R. parkeri* (denoted by arrows) are found in macrophages (**A**) and neutrophils (**B**). Bars = 5 μm.

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 (Fig. 5).

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 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 4 d 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, intact 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). While it was not definitively determined if these intracellular rickettsiae were alive or dead with the current assays, they were found whole with

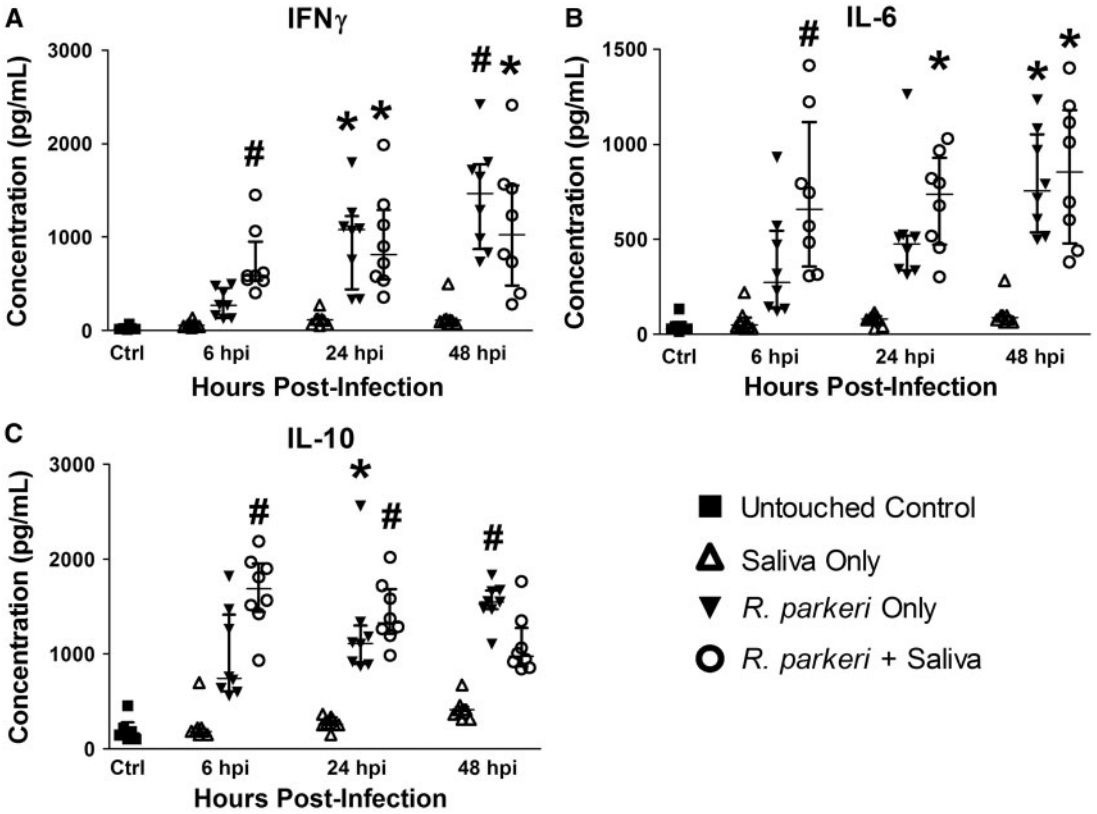

Fig. 4. Concentrations of skin inoculation site inflammatory cytokines are increased in response to intradermal *R. parkeri* inoculation, but not significantly altered by the addition of *A. maculatum* saliva to the rickettsial inoculum. Concentrations of interferon γ (**A**), interleukin-6 (**B**), 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).

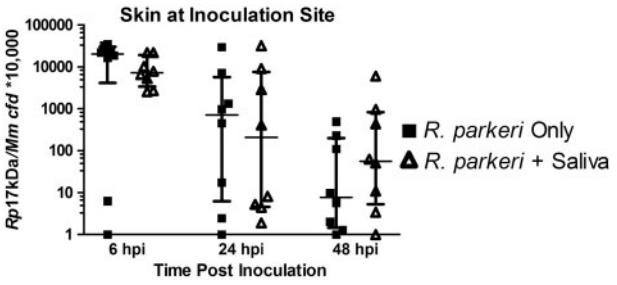

Fig. 5. *A. maculatum* saliva did not significantly alter *R. parkeri* numbers in inoculation site skin in the acute phase after inoculation. Rickettsial load as detected by qPCR in skin samples at the indicated time points is presented as *R. parkeri* 17 kDa copies per *Mus musculus cfd* copies times 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.

morphology similar to viable bacteria. 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. This inhibition is thought

to be of benefit to the tick as the influx of these cells to the tick feeding site may elicit pain, itch, disrupt blood flow, and cause direct damage to the feeding tick leading to host rejection of the tick (Kotal et al. 2015). One of the limitations of this study is that the panel of markers used (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, alternate markers, such as Langerin or DC-sign, or a different skin disruption protocol may be needed for further studies of cutaneous dendritic cells via flow cytometry.

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 (e.g. concavalin A), bacterial components (e.g. 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, 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 (Pechova et al. 2004). It is possible that tick modulation of the local cytokine milieu at the feeding site, may not be as important to facilitate tick feeding as alteration of immune cell recruitment, since these cytokines play more of a systemic role in immunity rather than altering the local immune response at the feeding site. 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, as the *R. parkeri* dose used in this study is a large dose, it may have overpowered the potential anti-inflammatory effects of *A. maculatum* saliva despite the fact that, as previously stated, it is within the range found in wild-caught ticks and similar to doses used in other studies. 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). Thus, it is possible that rickettsial proliferation was not seen in the C3H/HeN mice due to their robust immune response, as opposed to the susceptible immunodeficient C3H/HeJ mice. Also, while not determined *in vivo*, the *in vitro* doubling time of SFG *Rickettsia* is reported to be 10–12 h (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 antiinflammatory 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. It has been shown that the *A. maculatum* and *R. parkeri* model results in an eschar (Banajee et al. 2015), in contrast to other tick and rickettsial models (e.g., *Dermacentor variabilis* and *Rickettsia rickettsii*). Whether the associated pathology is tick or rickettsial dependent, the observations of the current study indicate that *A. maculatum* saliva is at least partially immunosuppressive at the inoculation site during the acute phase of *R. parkeri* infection, which may have downstream effects on the course of the rickettsial infection. While this study evaluated the effect of tick saliva on the acute immune response to *R. parkeri* infection, other phases of the immune response are also likely modulated. Further study should be performed to determine if the 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.

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