EXTENDING A FLOW CYTOMETRIC ASSAY TO ASSESS *RICKETTSIA PARKERI* INFECTIVITY IN SKIN-INFILTRATING LEUKOCYTES

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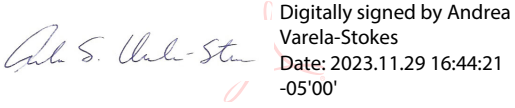

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Extending A Flow Cytometric Assay to Assess *Rickettsia parkeri* Infectivity in Skin-Infiltrating Leukocytes

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Abbreviations:

SFGR = Spotted fever group *Rickettsia* spp.

SFR = Spotted fever rickettsiosis

FCM = Flow cytometry

ABSTRACT:

Rickettsia parkeri is a tick-borne obligate intracellular pathogen in the spotted fever group Rickettsia spp. (SFGR) that causes spotted fever rickettsiosis (SFR). Our lab studies SFR using the guinea pig model and *R. parkeri* transmission by its primary vector, the Gulf Coast tick. Here, we expand a previously developed flow cytometric (FCM) assay for immunophenotyping cellular infiltrates in guinea pig skin to identify host cells targeted by *R. parkeri*. Specifically, we are testing an anti-SFGR antibody designed for immunohistochemistry (IHC) in an in vitro FCM experiment using differentiated THP-1 cells (human leukemia monocytic cell line) prior to moving to an animal study. First, we determined the number of THP-1 cells required to seed a 6-well plate to recover 1 x 10^6 viable differentiated cells per well. Then, we confirmed the percentage of formaldehyde required to inactivate *R. parkeri* when fixing cells in the FCM protocol. Next, we are determining the minimum time necessary for *R. parkeri* (MOI of 10) to invade host cells. We will apply those data to identify the multiplicity of infection (MOI) required to infect approximately 20 percent of the host cells for testing our FCM assay. We labeled the anti-SFGR antibody with the fluorophore CF405L in-house and are performing a titration to tell us if the antibody would be useful for flow cytometry and, if so, at what optimal dilution. We anticipate expanding our FCM assay to detect infected leukocytes in spiked guinea pig blood before moving to in vivo studies. Ultimately, this work will allow us to better understand disease progression and pathogenesis during infection after natural tick transmission in the guinea pig model.

Keywords

Spotted fever rickettsiosis

Guinea pig

Tick-bite

Amblyomma maculatum

THP-1 cells

Flow cytometry

1. Introduction

Rickettsia parkeri is an obligate, gram-negative, intracellular pathogen that belongs to the spotted fever group *Rickettsia* spp. (SFGR). It causes Spotted Fever Rickettsiosis (SFR) in humans, with disease in other species remaining unclear though the pathogen has been detected in species including dogs (Grasperge et al., 2012). Humans with SFR present with flu-like symptoms including fever, gastrointestinal problems, headache, vomiting and rashes. *Rickettsia parkeri* was first reported in 2004 (Paddock et al., 2004) for causing a mild form SFR compared to RMSF. SFR is transmitted via the bite of infected ticks, primarily by *Amblyomma maculatum*, also known as the Gulf Coast tick and found in the eastern United States. Following a bite by an infected tick, immune cells, including dendritic cells, macrophages, and lymphocytes respond by infiltrating the skin around the tick bite where the bacteria are initially located. However, the acute, local immune response to tick-transmitted rickettsiae is poorly understood.

We initiated this project using a flow cytometry (FCM) assay that was developed in 2021 to detect the leukocytes in guinea pig skin samples (Cross et al., 2021). Considering the immune cells infiltrating a tick bite site will be among the first cells to encounter the pathogen, *R. parkeri,* our goal was to expand our skin FCM assay to also detect *R. parkeri*-infected cells. We approached this by adapting an anti-SFGR antibody originally developed for immunofluorescent antibody testing. Here, we present our initial steps to determine feasibility of the antibody for FCM. Once optimized, the FCM assay will be used on skin samples and blood in our guinea pig model of SFR, monitoring infection of endothelial and immune cells encountered by rickettsiae during tick transmission.

2. Materials and Methods

2.1 *Inactivation of* R. parkeri *for FCM instrument.*

First, the percent formaldehyde needed to inactivate *R. parkeri* was determined. *Rickettsia parkeri* was cultivated in Vero cell cultures (African Green Monkey kidney epithelial cells) until 80% of Vero cells were infected with *R. parkeri* (determined by gross cytopathic effect followed by stained cytopins). Infected Vero cells were harvested, divided in five tubes, washed twice with phosphate buffered saline (PBS, pH 7.4) and then resuspended in PBS with 0%, 1%, 1.5%, 2%, and 3% formaldehyde and incubated at 30 min at 4℃ (mimicking a FCM assay protocol for cell preparative). After the incubation period, infected cells were pelleted by centrifugation (300 *x g* for 5 min) and resuspended in complete media (MEM with 10% FBS) then inoculated into three flasks (per formaldehyde treatment) with confluent layers of Vero cells. After 7 days, we examined the co-cultures using stained cytospins to determine the percentage of cells infected with *R. parkeri,* indicating live organism.

2.2 *Determining THP-1 cell concentration for cell plate.*

We determined the THP-1 cell concentration needed for recovering 1 x 10^6 total differentiated cells per well of a 6-well cell culture plate to obtain a sufficient number of cells for FCM analyses. THP-1 cells were maintained in vented flasks at concentrations of 1 x 10^5/mL – 1 x 10^6/mL using RPMI + 10% FBS + 1 µL/mL each of 2-Mercaptoethanol and Gentamicin at 37°C in 5% CO_2. THP-1 cells were seeded in 6-well cell culture plates with maintenance media and differentiated using 10 µM Phorbol-12-myristate-13-acetate

(PMA). After 48 hours, media was replaced to remove PMA and Gentamicin, and cultures incubated for another 24 hr. Cells were recovered using TrypLE Express and counted using a Cellometer Auto 2000 (Nexcelom) Cell Counter.

2.3 *Flow Cytometry Trial*

After determining the number of cells needed to seed wells of a 6-well plate to recover ~ 1 x 10^6 cells/well of differentiated THP-1 cells, we performed FCM on differentiated THP-1 cells infected with *R. parkeri. Rickettsia parkeri* was cultivated in Vero cells, then harvested to mechanically disrupt host cells and release rickettsiae, which were collected, filtered through a 3 µm filter to remove cell debris, and washed through a series of centrifugation steps with sterile PBS and 10,000 *x g* centrifugation. Enriched rickettsiae, in the Vero cell-free preparation, were inoculated in THP-1 seeded wells of the 6-well plate at an MOI of ~10-20. Once inoculated with rickettsiae, plates were centrifuged and then incubated at 37° with 5% CO_2 for 2 hours. For cell preparation for FCM, we used TrypLE Express Enzyme to remove cells, obtained a cell count, and determined % infected using a stained cytospin. Harvested cells were then incubated with the anti-SFGR antibody (Fuller Laboratories) labeled in-house using CF-405L. When the first trial did not reveal cells with *R. parkeri*, a second FCM trial was performed with the anti-SFGR antibody labeled with CF594 and an MOI of ~100. The FCM protocol followed that for skin cell preparation, including controls, published in Stokes et al. (2022).

2.4 *Indirect Fluorescent Antibody Test (IFA) for Antibody Activity*

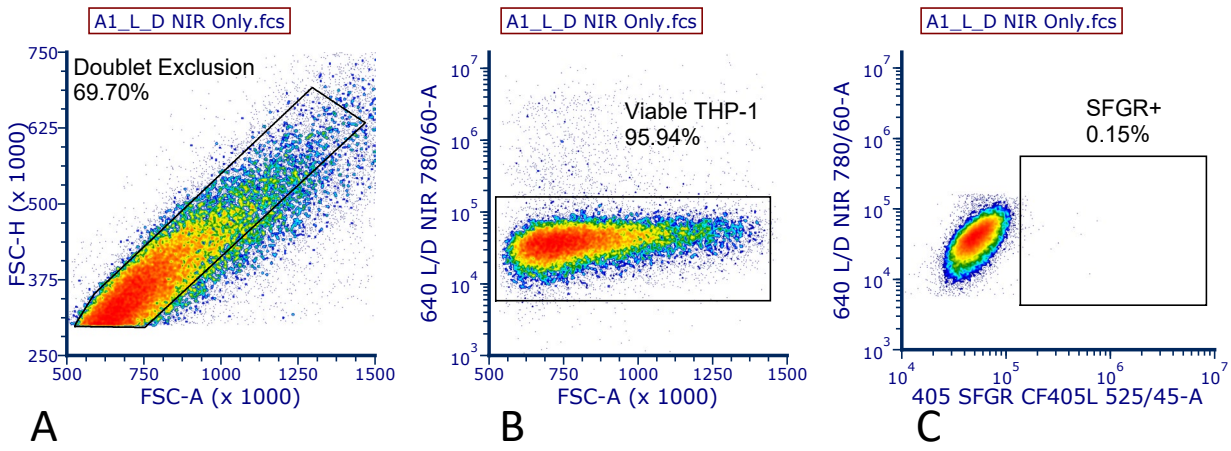
The purpose of this procedure was to evaluate the SFGR antibody activity for binding to *R. parkeri* as activity may have been lost due to prior freeze-thaws. Four 5-well microscope slides were coated with *R. parkeri* whole-cell antigen and allowed to dry. Slides were treated as follows: (1) no fixation; or fixation at 30 min at 4°C with (2) 1% formaldehyde, (3) 2% formaldehyde, or (4) 4% formaldehyde. Then wells on each slide were subjected to treatments as follows: (1) no primary or secondary antibody; (2) primary anti-SFGR antibody, no secondary antibody; (3) no primary, anti-mouse secondary antibody labeled with Alexa Fluor 488; (4) both anti-SFGR antibody and secondary labeled antibody; and (5) not used. The slides were incubated at 37°C for 20 min and washed in PBS followed by water between incubations with antibodies. After drying, we applied VectaShield and examined slides by microscopy for evidence of labeled SFGR (*R. parkeri*) using LED epifluorescence.

3. Results and Discussion

In comparison to the control group (0% formaldehyde), in which Vero cell flasks were heavily infected with both intracellular and extracellular *R. parkeri,* we found that flasks inoculated with *R. parkeri* that were fixed with 2% and 3% formaldehyde did not survive exposure – Vero cells were also killed. However, infected cells were not observed with 1.5% formaldehyde-fixed *R. parkeri* and few Vero cells with possible *R. parkeri* could be observed in flasks inoculated with 1% formaldehyde-fixed *R. parkeri.* Thus, our FCM assay cell preparation protocols that used 1% formaldehyde or above were deemed safe to use in the flow cytometer.

When preparing THP-1 cell plates, we determined that 2 x 10^6 THP-1 cells per well were needed for a recovery of 1 x 10^6 differentiated cells in each well using TrypLE. Thus, we proceeded with this THP-1 cell concentration in our FCM trials. During the first FCM trial with CF405-labeled antibody, no infected cells were detected (Fig. 1). After switching to a brighter label CF594 in the second trial, the results improved from the first trial (not shown). However, we became concerned that the antibody may have lost activity after freeze-thaw opportunities during a lab relocation. The immunofluorescent antibody test demonstrated evidence of staining in the wells that had both primary and secondary antibody, and were fixed with 1%, 2%, or 4% formaldehyde, confirming the antibody was active, though the level of activity could not be discerned and no differences were noted in the intensity of staining between slides with the excepted of the “no fix” slide in which no staining was observed.

Fig 1. (A-C) Gating strategy for negative control *R. parkeri*-infected THP-1 cells where sample preparation excluded CF406L-labeled SFGR antibody. (A) Eliminated doublets; (B) eliminated remaining dead THP-1 cells; (C) false positive cells. (D-F) Gating strategy for *R. parkeri*-infected cells *including* CF405L-labeled SFGR antibody. (D) Eliminated doublets; (E) eliminated remaining dead THP-1 cells; (F) positive cells where below limit.



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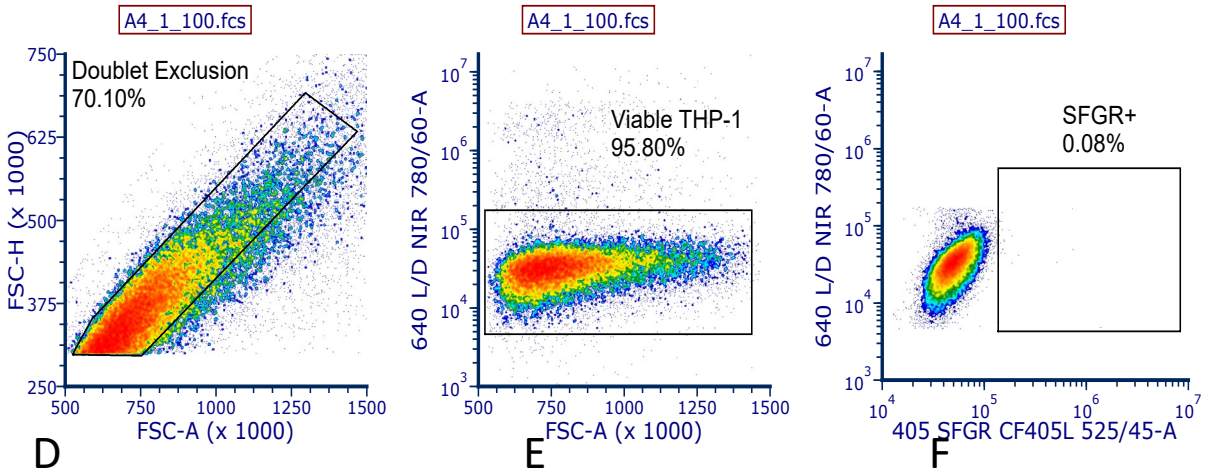

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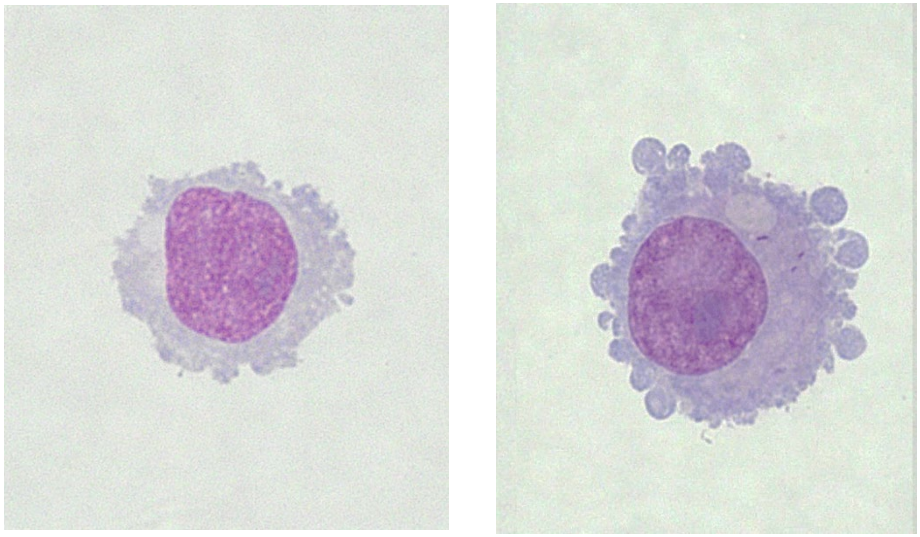
165 While we confirmed that 1% formaldehyde was enough for *R. parkeri* inactivation, we also
166 determined that 2% formaldehyde would be more suitable for cell preparation for the FCM
167 assay. We considered that the CF405 label might not be bright enough for detection in
168 the first FCM trial. The THP-1 cells used in the second trial had a higher number of
169 rickettsial rods observable in stained cytospin preparations reflecting the higher MOI; we
170 were able to detect cells that had more than 10 intracellular *R. parkeri* (Fig. 2). However,
171 the FCM results were still somewhat equivocal and we were unable to easily distinguish
172 a population of infected cells.

173

Fig. 2. Wright-Giemsa stained cytopsins of THP-1 cells used in FCM Trial 1. (A) Uninfected THP-1 cell; (B) *R.* parkeri-infected THP-1 cell (arrows at *R. parkeri*).



While the second trial showed promise, we believe we need to obtain new SFGR antibody that has not undergone multiple freeze-thaw episodes, determine the optimal time of THP-1 infection (may not be 2 hrs) and the optimal MOI to obtain a THP-1 cell preparation with 10-20% as this will allow us to best identify separate infected and uninfected THP-1 cell populations by FCM. Once we optimize the antibody for FCM, we will be able to apply our FCM assay for evaluating *R. parkeri* immune cell tropism in the skin after tick transmission of *R. parkeri* using the guinea pig model. Ultimately, this will improve our understanding of the acute immune response to tick-transmitted *R. parkeri* and disease progression in the guinea pig model of SFR.

4. Conclusion

Our study provided foundational data, including the requisite formaldehyde percentage for pathogen inactivation and the optimal concentration of THP-1 cells for plating, to pursue expansion of an FCM assay to detect *R. parkeri*-infected immune cells. Subsequent experiments should confirm the ideal time for infection and MOI for optimizing the flow cytometry (FCM) assay as part of optimizing the FCM assay protocol and prior to applying this assay to studies using the guinea pig-tick-*Rickettsia* system.

5. Acknowledgments

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6. Conflict of Interest Statement

The authors declare no conflict of interest.

7. Author Contributions

XY performed the majority of the experiments and wrote the first draft of the manuscript; JVS generated the ideas and designed experiments with AVS and reviewed the manuscript; AVS generated the ideas and designed experiments with JVS and assisted with experiments and writing drafts.

8. References

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