ASR 31st Meeting
Greenville, SC
June 25-28, 2022

Advances in the Biological & Molecular Mechanisms of Rickettsial Pathogenesis

Thanks to our Sponsors!

Funding for this conference was made possible (in part) by R13AI169930 from the National Institute of Allergy and Infectious Diseases. The views expressed in written conference materials or publications and by speakers and moderators do not necessarily reflect the official policies of the U.S. Dept of Health and Human Services; nor does mention of trade names, commercial practices, or organizations imply endorsement by the U.S. Government.

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WELCOME

Dear ASR Members,

Having been almost three years since we last met, I am delighted to welcome back our regular attendees and meet our new members. I appreciate your willingness to stay involved with the virtual sessions we hosted, it was a great opportunity to connect and hear about research advances in the field.

The time off from in-person meetings allowed me to reflect on what I enjoy most about this society. I find this meeting to be just the right size to connect with my colleagues. The ASR format allows for concentrated intellectual exchange relative to the biological interactions of a variety of bacteria with their hosts (vertebrate and invertebrate). The diversity of research for a group this size is pretty spectacular. Likewise, as this has been my primary society for more than two decades, the progress the field has made both technically and conceptually is inspiring. In addition to the science, I can speak to the supportive nature of ASR. The society has consistently focused on the next generation of researchers through presentation opportunities, monetary support, and mentoring of early career colleagues. This year will be no different.

As we gather this year, I look forward to seeing old friends again, making new ones, and participating in a meeting that advances understanding of rickettsial biology and its impact on host-vector-rickettsiae interactions.

Thank you for the opportunity to serve as the ASR President.

Kevin Macaluso
ASR President

AGENDA

Saturday, June 25

12:00 – 7:00 PM **ASR Registration Desk** **Regency Foyer**
Sheilah Jewart

3:00 PM **Day One: Welcome & Introductions** **Regency Ballroom**
Kevin Macaluso, President

Session 1: Advances in clinical diagnostics, pathogen discovery, and vaccines for rickettsial diseases.
Chair: Diana Scorpio

3:05 PM 1A. Plenary overview: Advances in laboratory diagnosis of rickettsial diseases at the acute stage of illness
Cecilia Kato (188)

3:30 PM 1B. Detection and isolation of *Rickettsia tillamookensis* (Rickettsiales: Rickettsiaceae) from *Ixodes pacificus* (Acari: Ixodidae) from multiple regions of California
Chris Paddock (205)

3:45 PM 1C. Targeted mutagenesis in *Anaplasma phagocytophilium* for modified live vaccine development
Jonathan Ferm (118)

4:00 PM 1D. Combined diagnosis of IFA and PCR increase the confirmation of suspected spotted fever cases
Liliane Duraes (173)

4:15 PM 1E. An antigen-based diagnostic lateral-flow-assay for acute spotted fever rickettsioses
Rong Fang (178)

4:30 PM 1F. Targeted Mutagenesis in *Anaplasma marginale* to define virulence and vaccine development against bovine Anaplasmosis
Roman Ganta (184)

4:45 PM Break

5:00 PM 1G. Mentoring Session: Transitioning to Early Career Stage Investigator
Lisa Brown, Lilian Crosby, Sean Riley
Rebecca Lamason, Dana Shaw

6:00-7:30 PM Welcome Reception – (2 Drink Tickets) Regency Foyer

6:30-7:00 PM ASR Historical Lecture Regency Ballroom
Roman Ganta

Sunday, June 26

6:30 – 9:00 AM **Buffet Breakfast – Give coupon to server** **Roost Restaurant**
Note: The restaurant has limited seating, so in order to serve every one breakfast in time for the meeting, please share tables and do not linger after you have finished your meal. Thank you!

8:00 – 5:00 PM **ASR Desk** **Regency Foyer**

8:30 AM **Day Two: Introductions** **Regency Ballroom**
Kevin Macaluso

Session 2: Tick-borne spotted fever group *Rickettsia*.
Chair: Sean Riley

8:35 AM 2A. Keynote: Rickettsial disease: The delicate balance of microbial fitness and human genetic diversity
Steve Dumler

9:20 AM 2B. Invited short talk: Probing *Rickettsia* pathogenesis using advanced biochemical and genetic tools
Rebecca Lamason (227)

9:40 AM 2C. Invited short talk: Selective fragmentation of the trans-Golgi apparatus by *Rickettsia rickettsii*
Ted Hackstadt (234)

10:00 AM 2D. Whole-genome sequencing of *R. amblyommatis* isolated from *A. americanum* in Long Island reveals unique genetic traits and phenotypes associated with mild rickettsioses
Hwan Kim (190)

10:15 AM 2E. Identification of transposon insertion mutants in pathogenic SFG *Rickettsia* species that are impaired in intracellular growth within mammalian phagocytic cells
Juan Martinez (196)

10:30 AM 2F. Classical complement activation, IgM, and anaphylatoxins shape the immune response to *Rickettsia* infection
Mustapha Dahmani (112)

10:45 AM **Break** **Regency Foyer**

Session 3: *Coxiella* biology and pathogenesis.
Chair: Dan Voth

11:00 AM 3A: Plenary overview: Lessons from an obligate stealth pathogen: Multiple ways to “skin a cat”
Jim Samuel (164)

11:30 AM 3B. Investigating the prophylactic and therapeutic potential of doxycycline using an A/J mouse aerosol model of Q-fever
Chris Jenkins (187)

11:45 AM 3C. *Coxiella burnetii* manipulates the type I interferon pathway in a T4BSS dependent manner
Erin van Schaik (217)

12:00 PM 3D. Development of a CRISPR interference system for genetic manipulation of the bacterial human pathogen *Coxiella burnetii*
Shaun Wachter (159)

12:15 PM 3E. *Drosophila* sting mediates *Coxiella burnetii* infection by regulating oxidative stress
Rosa Marena Guzman (121)

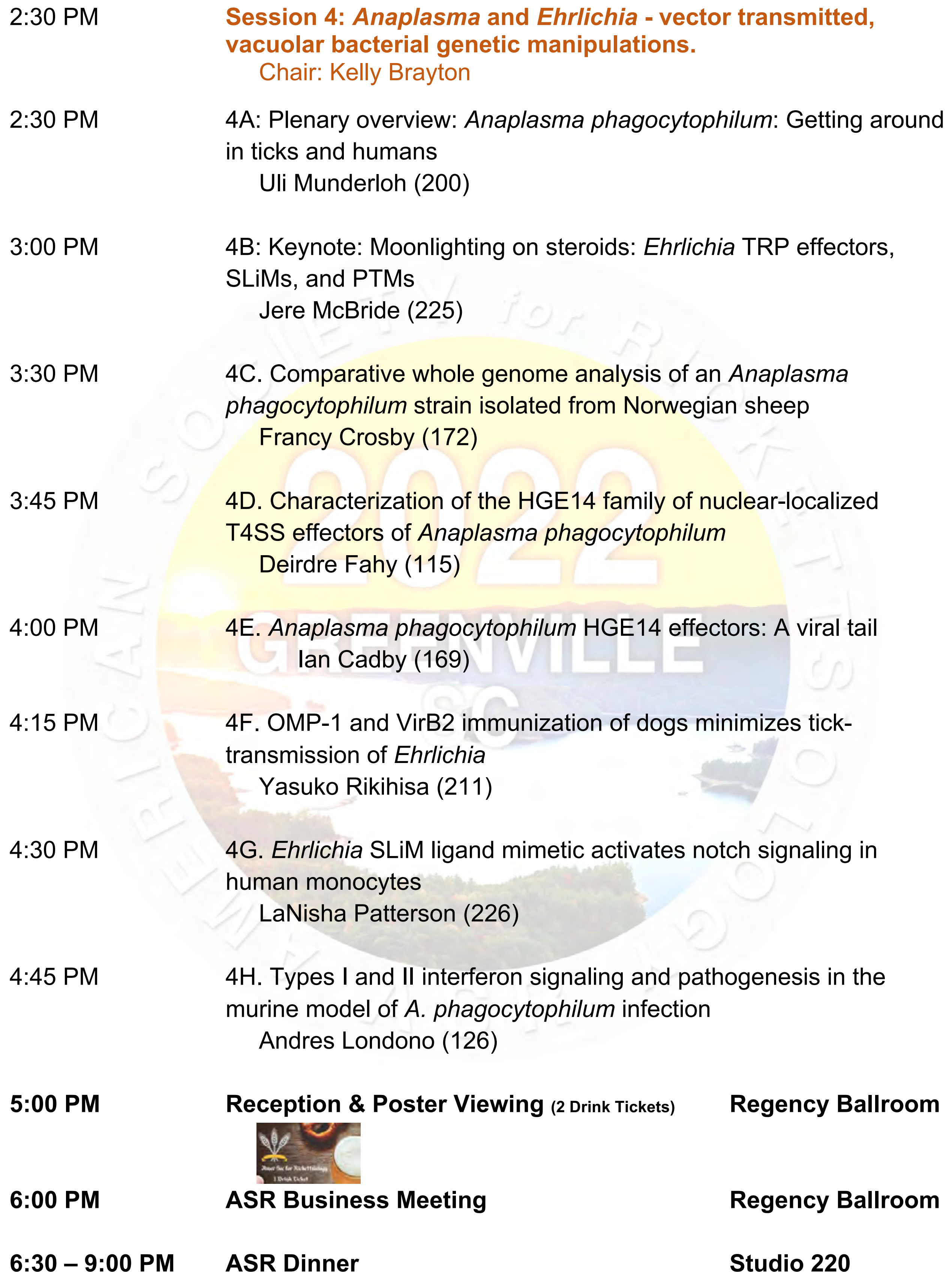
12:30 PM 3F. Identification and functional analysis of *Coxiella burnetii* dugway strain-specific type IV secretion system effector proteins
Mahelat Tesfamariam (151)

12:45 PM **Lunch** **Regency Foyer**
Note: Pick up your buffet lunch and take back into the meeting room.

1:30 PM **Poster Session I – Even #s** **Regency Ballroom**

Poster#	***Poster Title***	**Last name**
104	The long non-coding RNA landscape of host cells during *Coxiella burnetii* infection	Arunima
106	*Ehrlichia chaffeensis* TRP120 targets nuclear innate immune regulator hnRNPA2B1 for degredation	Bui
108	*Ehrlichia* SLiM ligand mimetic activates hedgehog signaling to engage a BCL-2 anti-apoptotic cellular program	Byerly
110	Host targeted drugs as an alternative control of *Rickettsia* infection	Cook
116	Efficacy evaluation of *Anaplasma centrale* Msp2 hypervariable regions for protection from bovine Anaplasmosis	Falghoush
120	The *A. phagocytophilum* T4SS tick effector contains EPIYA motifs and directs tyrosine phosphorylation	Genera
122	An investigation of relationship between *Wolbachia* endosymbionts and *Rickettsia felis* in the cat fleas, *Ctenocephalides felis*	Jirakanwisal
128	*Coxiella burnetii* requires type IVB secretion system to suppress host TLR3/TRIF-dependent NF-kB-activation	Mahapatra
130	Hemocyte-mediated immune response to a bacterial infection in the cat flea (*Ctenocephalides felis*)	Muñoz
132	Regulator of actin-based Motility (RoaM) downregulates actin tail formation by *Rickettsia rickettsii* and is negatively selected in mammalian cell culture	Nock
134	TNF-a-dependent C-type lectin Mincle plays a significant role in innate immune recognition in the brain during scrub typhus infection in both outbred CD-1 and inbred C57BL/6 mice	Onyoni
136	*Ehrlichia chaffeensis* activation of notch signaling stabilizes XIAP expression to inhibit caspase activation	Patterson
138	Investigation of *Ehrlichia chaffeensis* TRP120 as a PGDF ligand mimic	Pittner
142	A pleiotropic metabolite affects microbial infection and arthropod vector fitness	Samaddar
144	Shaping the understanding of the unfolded protein response and vector competency in *Ixodes scapularis*	Sidak-Lofits
146	Quantitative analysis of growth and morphology in the obligate intracellular pathogen *Rickettsia parkeri*	Smith
148	CteG mediated control of centrin-2: Getting to the center of how chlamydia manipulates the centrosome cycle	Steiert
150	Role of Sca4 in the dissemination and transmission of *Rickettsia parkeri* in *Amblyomma maculatum*	Suwanbongkot
152	Host transcriptomic profiling of CD-1 outbred mice with severe clinical outcomes following infection with *Orientia tsutsugamushi*	Thiriot
154	Roles of rickettsial outer membrane protein B (OmpB) in tick vector infections	Tongluan

156	Novel detection of *Rickettsia* species in *Amblyomma maculatum* found in Callaway County, Missouri	Vaughn
160	Analysis of a new type IV secretion system effector of *Anaplasma phagocytophilum*	Wang
162	Comparative transcriptomics identifies *Coxiella burnetii* survival strategies in continuous axenic media growth	Yadav
166	An *in vitro* antibiotic treatment algorithm to eliminate *Mycoplasma* contamination from rickettsial cultures	Allerdice
168	Investigating interferon signaling as a key determinant of human spotted fever disease	Burke
170	*Anaplasma phagocytophilum* transmission activates interferon signaling pathways in the skin	Chavez
174	The insect *Galleria mellonella* as an infection model to investigate pathogenesis of *Rickettsia*	Eremeeva
176	UK outbreaks of Q fever: Strain characterisation and assessment of aerosol survival	Essex-Lopresti
180	Ectoparasite profile and *Rickettsia* burden in small mammals collected in central Pennsylvania	Fedrow
182	Bovine anaplasmosis prevalence assessed in beef cattle from diverse geographic regions of California	Fitzwater
186	Multiple *Ehrlichia chaffeensis* genes critical for its persistent infection in a vertebrate host are nonessential for its growth in the tick vector*, Amblyomma americanum*	Jaworski
192	Inhibition of *Ehrlichia chaffeensis* infection by cell-permeable bicyclic peptides that bind *Ehrlichia* type IV secretion effector Etf-1	Lin
194	Q fever post-vaccination hypersensitivity modeling reveals sex dimorphism and a less reactogenic vaccine candidate	Long
198	Investigation of genotype variations among *Orientia tsutsugamushi* human isolates as possible predictors of severe scrub typhus	Mullins
202	Proof-of-concept efficacy of doxycycline in an inhalational marmoset model of Q Fever	Nelson
204	*Anaplasma marginale* enters tick cells using clathrin and proteins of the Msp1 superfamily	Noh
208	Examining the evolution of pathogenicity in *Coxiella* to identify novel therapeutic targets	Raghavan
210	The IMPACT of *Coxiella*: Learning from a pathogen during a pandemic	Reed
212	The interplay between flea-borne rickettsiae	Richards
216	Assessment of the pathogenicity of purportedly nonpathogenic *Rickettsia* via tick bite from naturally infected vectors	Snellgrove
220	Expression of *Orientia tsutsugamushi* p56 antigen in recombinant non-replicative adenovirus virus vector for evaluation of protection against scrub typhus infection	Walker



2:30 PM — **Session 4: *Anaplasma* and *Ehrlichia* - vector transmitted, vacuolar bacterial genetic manipulations.**
Chair: Kelly Brayton

2:30 PM — 4A: Plenary overview: *Anaplasma phagocytophilum*: Getting around in ticks and humans
Uli Munderloh (200)

3:00 PM — 4B: Keynote: Moonlighting on steroids: *Ehrlichia* TRP effectors, SLiMs, and PTMs
Jere McBride (225)

3:30 PM — 4C. Comparative whole genome analysis of an *Anaplasma phagocytophilum* strain isolated from Norwegian sheep
Francy Crosby (172)

3:45 PM — 4D. Characterization of the HGE14 family of nuclear-localized T4SS effectors of *Anaplasma phagocytophilum*
Deirdre Fahy (115)

4:00 PM — 4E. *Anaplasma phagocytophilum* HGE14 effectors: A viral tail
Ian Cadby (169)

4:15 PM — 4F. OMP-1 and VirB2 immunization of dogs minimizes tick-transmission of *Ehrlichia*
Yasuko Rikihisa (211)

4:30 PM — 4G. *Ehrlichia* SLiM ligand mimetic activates notch signaling in human monocytes
LaNisha Patterson (226)

4:45 PM — 4H. Types I and II interferon signaling and pathogenesis in the murine model of *A. phagocytophilum* infection
Andres Londono (126)

5:00 PM	**Reception & Poster Viewing** **(2 Drink Tickets)**	**Regency Ballroom**
6:00 PM	**ASR Business Meeting**	**Regency Ballroom**
6:30 – 9:00 PM	**ASR Dinner**	**Studio 220**

Monday, June 27

6:30 – 9:00 AM **Buffet Breakfast - Give coupon to server** **Roost Restaurant**

8:00 – 1:30 PM **ASR Desk** **Regency Foyer**

8:30 AM **Day Three: ASR Annual Meeting** **Regency Ballroom**

Session 5: Bacterial effectors and response regulators.
Chair: Rahul Raghav

8:30 AM 5A. A transposon screen to identify potential essential genes in *Coxiella burnetii*
Georgie Metters (197)

8:45 AM 5B. Investigating mechanisms by which spotted fever group (SFG) rickettsiae induce microvascular endothelial cell barrier permeability
Jennifer Farner (117)

9:00 AM 5C. The CD36 homolog croquemort binds infection-derived lipids and initiates antibacterial immunity in *Ixodes scapularis*
Anya O'Neal (133)

9:15 AM 5D. Hacking the tick: Identification and characterization of a tick-targeted effector from *Anaplasma phagocytophilum*
Jason Park (206)

9:30 AM 5E. Pathogenic *Rickettsia* species manipulate host immune defense pathways to facilitate intracytosolic replication within the host
Oliver Voss (218)

9:45 AM **Poster Session 2 – Odd #s**

Poster#	*Poster Title*	Last name
103	Deciphering the role of *Amblyomma maculatum* hemocytes during *Rickettsia parkeri* infection	Adegoke
105	*Coxiella burnetii* as an expression vector for SARS-CoV-2 spike protein: A tool for the study of host-pathogen interactions	Binette
107	Rab27 in tick extracellular biogenesis and infection	Butler
109	Mechanisms of *Coxiella burnetii* whole cell vaccine reactogenicity	van Schaik
111	Identification of differentially expressed genes in *Ixodes scapularis* cells involved in vector-pathogen interactions during early *Anaplasma phagocytophilum* infection	Cull
113	Functional characterization of a cat flea salivary antigen	Danchenko
119	RNA analysis of *Ehrlichia chaffeensis* 7 genes spanning from ECH_0659 to ECH_0665 encoding for several phage related proteins and their orthologs in related *Anaplasma* and *Ehrlichia* species	Genda
125	*Anaplasma phagocytophilum* AipA interacts with CD13 to facilitate host cell invasion	Lind
127	The canine host serving as a sentinel species for tick-borne diseases caused by *Anaplasma*, *Ehrlichia*, and *Borrelia* pathogens impacting human health in the USA	Madesh
129	Tick regulation of epidermal skin immunity facilitates an advantageous arthropod feeding environment	Marnin
131	*Anaplasma phagocytophilum* recruitment and infection of neutrophils in a 3D model of human dermal microvessels.	Nenortas
133	The CD36 homolog Croquemort binds infection-derived lipids and initiates antibacterial immunity in *Ixodes scapularis*	O'Neal
135	The impact of caspase-8 and STING interactions on extrinsic apoptosis during *Coxiella burnetii* infection	Osbron
137	Evaluation of qPCR assay using target gene containing repetitive sequences for *Rickettsia typhi* detection in patients with acute febrile illness	Phuklia
139	Tick hemocytes at the single cell level	Rolandelli
141	*Rickettsia* species secret effector(s) that modulates intracellular trafficking to establish a replicative niche in host cytosol	Sadik
143	Omics-derived evidence that *Orientia tsutsugamushi* both pirates and modulates host central metabolism during infection	Sanchez

145	Investigation of the potential involvement of RIPK3 in *Orientia tsutsugamushi* inhibition of NF-κB	Siff
147	*Ehrlichia chaffeensis* TRP120 activates BMP2 signaling in monocytes	Solomon
149	The attraction of *Dermacentor variabilis* to road edge habitat: Evaluating the role of sensory organs in the distribution of American dog ticks in the field	Stewart
155	Tissue-specific transcriptional responses to *Rickettsia rickettsii* infection in mice	Towey
157	Cryptic genes for interbacterial antagonism distinguish *Rickettsia species* infecting blacklegged tick from other *Rickettsia* pathogens	Verhoeve
161	Symbiont control of arthropod vector autophagy: Insights from the blacklegged tick, *Ixodes scapularis*	Wang
163	Ca2+ regulation during host-*Rickettsia* interaction	Zhu
165	Downregulated expression of MHC-I in *Anaplasma phagocytophilum*-stimulated human monocyte-derived macrophages: A role for NLRC5 isoforms?	Alharthi
167	Rickettsioses identified as a cause of febrile illness requiring hospitalization at two hospitals in Uganda	Blair
171	Genomes of *Rickettsia rickettsii* strains representative of areas with elevated Rocky Mountain spotted fever risk	Clark
175	Utility of next-generation sequencing for etiological diagnosis of rickettsial diseases	Eremeeva
177	*Rickettsia conorii* disrupts inter-microvascular endothelial cell tight junction via activating NLRP3 inflammasome	Fang
179	A single dose immunization of live-attenuated *R. parkeri* mutant confers complete protection against two fatal rickettsioses in mice	Fang
183	IgG and IgM antibody reactivity with individual Spotted Fever *Rickettsia* antigens	Fuller
185	Endothelial exosome plays functional role during rickettsial infection	Gong
191	Importazole, a specific inhibitor of importin-dependent nuclear transport, suppresses growth of *Anaplasma phagocytophilum* in HL-60 cells by preventing AnkA nuclear accumulation	Kim
193	EcxR is a global gene regular which interacts with the promoter segments of multiple *Ehrlichia chaffeensis* genes transcribed by RNA polymerase holoenzyme containing the housekeeping or alternative sigma factors	Liu
195	*Ehrlichia canis* immunome is dominated by hypothetical proteins and conformation-dependent antibody epitopes	Luo

199	Role of lipid droplets and prostaglandinE2 in *Coxiella burnetii* intracellular growth	Mulye
201	A small non-coding RNA mediates transcript stability and expression of cytochrome bd-I ubiquinol oxidase subunit 1 in *Rickettsia conorii*	Narra
207	Evaluation of *Ehrlichia canis* diagnostic protein immunoreactivity and dual host expression	McBride
209	The *Coxiella* effector CvpG modulates fusion properties of the *Coxiella* containing vacuole	Reed
213	Endothelial mTOR activation with *R. rickettsii* strains of varying virulence: Possible roles in the modulation of autophagy, inflammation, and rickettsial replication	Narra
215	The retropepsin-type protease APRc as a novel Ig-binding protein and moonlighting immune evasion factor of *Rickettsia*	Simões
221	Type VIB secretion system protein, icmE contribute to *Coxiella burnetii* virulence, intracellular replication, and activation of inflammasome mediators during infection	Zhang
223	*Ehrlichia chaffeensis* TRP120-mediated ubiquitination and degradation of transcriptional corepressor TLE4	Zhu
229	Cat flea coinfection with *Rickettsia felis* and *Rickettsia typhi*	Laukaitis
231	Potential vaccine targets in VirB10 of the Anaplasmataceae	Crosby
233	An insight into the epigenetics of *Ixodes scapularis* populations	Chavez

Session 6: *Orientia* biology and pathogenesis.
Chair: Allen Richards

10:45 AM — 6A: Plenary overview: The intracellular lifecycle of *Orientia tsutsugamushi*
Jeanne Salje (230)

11:15 AM — 6B: Invited short talk: *Orientia tsutsugamushi* host-pathogen interactions and new tools for discovery
Paige Allen (224)

11:45 AM — 6C. Altered lethality associated with cytokine (IFNγ, TNF, and IL2) depletion in a non-lethal murine model of *Orientia tsutsugamushi* infection (compared to lethal and sublethal models)
Alison Fedrow (181)

12:00 PM	6D. Recognition of Vita-PAMPs in *Orientia tsutsugamushi* by endosomal RNA receptors Christian Keller (189)
12:15 PM	6E. *Orientia tsutsugamushi* effector Ank5 decreases surface MHC-I levels by targeting the transcriptional activator NLRC5 for proteasomal degradation Haley Adcox (102)
12:30 PM	6F. A murine model of waning scrub typhus cross-protection between heterologous strains of *Orientia tsutsugamushi* David Walker (219)
12:45 PM	NIH Roundtable - pick up box lunch **Regency Foyer** **Ticket required – Must be pre-registered**
1:00 PM	**NIH Roundtable Discussion for Trainees & Early Career Investigators** Liangbiao Zheng and Sam Perdue
	Afternoon / Evening Free – Enjoy Greenville!
2:00 – 3:00 PM	**Swamp Rabbit Bicycle Trail** Bike rental $25 includes helmet and lock (4 hrs) Pick up Bikes @ Reedy Rides – 50 South Richardson Street

The Swamp Rabbit Trail is a self-guided 22-mile multi-use (walk & bike) greenway that traverses along the Reedy River, an old railroad corridor and through Greenville City parks.

Breweries in walking distance from the Hyatt:

1. Fireforge Crafted Beer
2. Iron Hill Brewery & Restaurant
3. Birds Fly South Ale Project
4. The Eighth State Brewing Company
5. 13 Stripes Brewery
6. 13 Stripes River Lodge
7. Liability Brewing Co
8. Pangaea Brewing Company
9. Carolina Bauernhaus Greenville
10. Thomas Creek Brewery
11. The Velo Fellow
12. Southernside Brewing Co.
13. Yee-Haw Brewing Company
14. Brewery 85

Tuesday, June 28

6:30 – 9:00 AM	**Buffet Breakfast - Give coupon to server**	**Roost Restaurant**
8:00 – 12:00 PM	**ASR Desk**	**Regency Foyer**
8:30 AM	**Day Four: ASR Annual Meeting**	**Regency Ballroom**

Session 7: Vector contribution to rickettsial pathogenesis.
Chair: Alyssa Snellgrove

8:30 AM — 7A: Plenary overview: Arthropod vectors: More than mobile syringes
Dana Shaw (214)

9:00 AM — 7B. Targeting tick transporter to prevent rickettsial pathogen transmission
Girish Neelakanta

9:30 AM — 7C. *Anaplasma marginale* infection of *Dermacentor andersoni* primary midgut cell culture is dependent on fucosylated glycans
Rubikah Vimonish (158)

9:45 AM — 7D. A pleiotropic metabolite affects microbial infection and arthropod vector fitness
Sourabh Samaddar (142)

10:00 AM — 7E. The use of the antigenically variable major surface protein 2 (msp2) in the establishment of superinfection during natural tick transmission of *Anaplasma marginale* in southern Ghana
Roberta Koku (123)

10:15 AM	**Break**	**Regency Foyer**

Session 8: Genomic determinants of rickettsial lifestyles.
Chair: Lisa Brown

10:45 AM — 8A. Plenary overview: *Drosophila melanogaster* as a chassis for understanding bacterial effector targets of arthropods
Irene Newton (140)

11:45 AM — 8B. *Coxiella burnetii* polysaccharide vaccine protects guinea pigs from coxiellosis
John Stenos (228)

12:15 PM — 8C. Sticking together – *Ixodes pacificus* and symbiotic *Rickettsia monacensis* strain Humboldt
Jianmin Zhong (222)

12:30 PM — 8D. Determining transmission determinants of an emerging flea-borne rickettsiosis
Hanna Laukaitis (124)

12:45 PM — Closing Remarks by 2023 President

We look forward to seeing you next year!

DELEGATES

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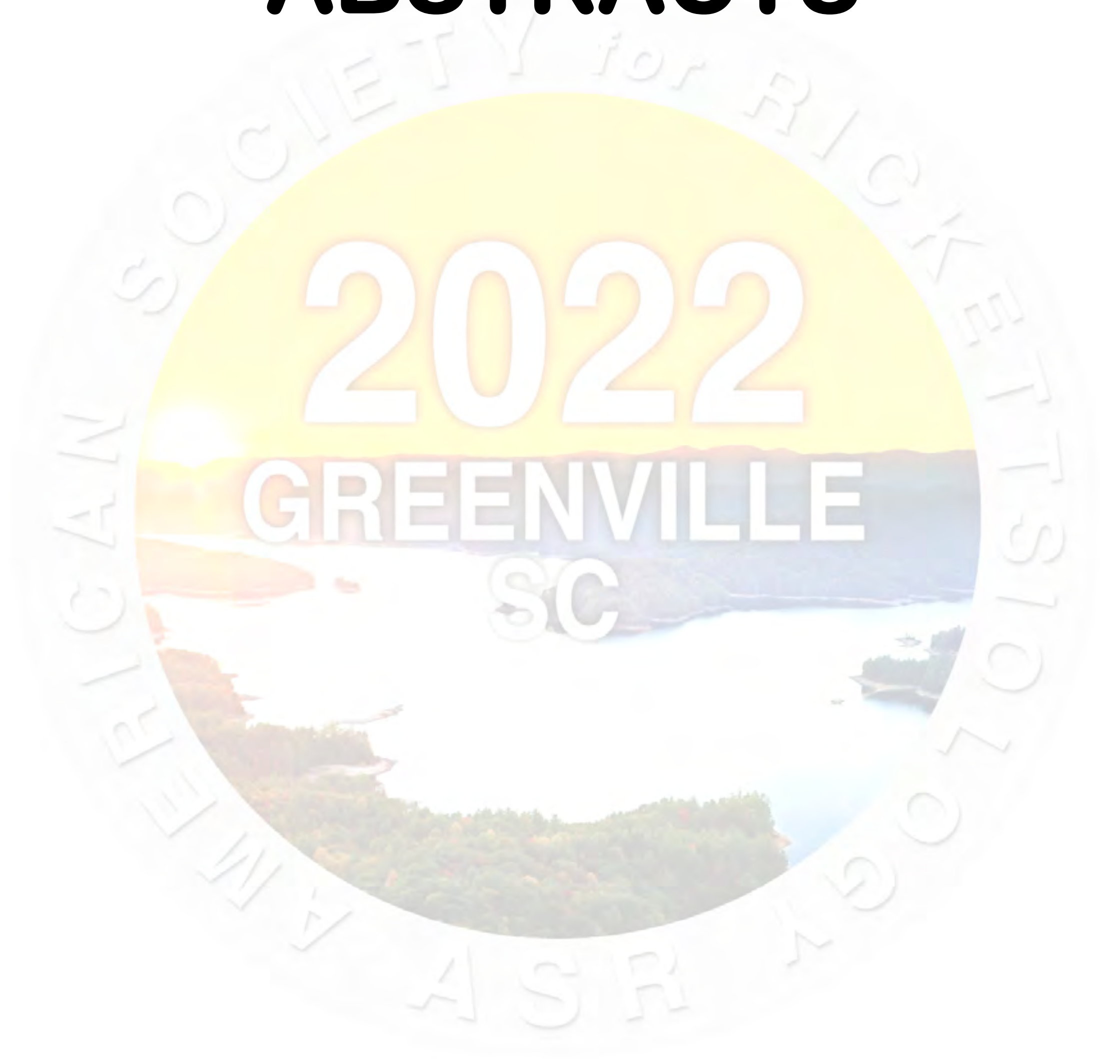
ABSTRACTS



Orientia tsutsugamushi effector Ank5 decreases surface MHC-I levels by targeting the transcriptional activator, NLRC5, for proteasomal degradation

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Orientia tsutsugamushi is an obligate intracellular bacterium and etiologic agent of scrub typhus, a potentially fatal disease with emerging global implications. Mechanisms facilitating *O. tsutsugamushi* pathobiology and immune response modulation are poorly understood. Adaptive immunity to intracellular pathogens relies on surface exposed MHC-I molecules to recognize infected cells. We previously reported that *O. tsutsugamushi* modulates the MHC-I pathway through targeted proteasome-dependent reduction of the MHC-I gene transactivator, NLRC5. *O. tsutsugamushi* encodes numerous T1SS Ank effectors that harbor eukaryotic-like ankyrin repeats (ARs). Most *O. tsutsugamushi* Anks also contain a C-terminal F-box domain capable of co-opting the host SCF complex that ubiquitinates target proteins for proteasomal degradation. Here we identify Ank5 as an effector responsible for NLRC5 degradation. Yeast two-hybrid identified NLRC5 as an Ank5 binding partner, an interaction that was confirmed by co-immunoprecipitation. Further co-immunoprecipitation assays performed using Ank5 proteins lacking one or more of its four ARs pinpointed that the interaction is dependent on AR4. Ectopically expressed Ank5 phenocopied infection-induced NLRC5 degradation in a manner that was proteasome-, F-box-, and SCF complex-dependent. Ank5 bearing a functionally inactivated F-box incapable of nucleating the SCF complex (Ank5-F-boxAAAAA) was unable to degrade NLRC5 yet still able to retain NLRC5 in the cytoplasm. Thus, Ank5 reduces cellular levels of MHC-I by two distinct but synergistic mechanisms: it sequesters NLCR5 in the cytoplasm and promotes its proteasomal degradation. To validate this model during infection, we used a competitive antagonist approach that circumvents *O. tsutsugamushi* genetic intractability. Host cells expressing GFP or GFP-Ank5- F-boxAAAAA were infected followed by measurement of NLCR5 levels. GFP-Ank5-F- boxAAAAA functioned as a dominant negative competitor of bacterial-derived Ank5 to prevent NLRC5 degradation. Overall, these data confirm that Ank5 is responsible for binding and degrading NLRC5 during *O. tsutsugamushi* infection resulting in reduced surface MHC-I levels.

Deciphering the role of *Amblyomma maculatum* hemocytes during *Rickettsia parkeri* infection

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Ticks are a diverse group of arthropod vectors that transmit a wide array of pathogens. The increase in tick-borne diseases is a significant threat to public health without preventive measures. The innate immune system of hematophagous ticks is divided into two primary mechanisms of immune defense: soluble effector molecules (such as antimicrobial peptides) that mediate humoral responses and cellular immune responses such as phagocytosis and encapsulation that promote pathogen killing. Immune cells, commonly called hemocytes, are a critical component of the immune system and produce immune factors that either facilitate or suppress pathogen development in a tick. Despite their importance in pathogen development, understanding of their basic biology and molecular mechanisms is limited. In this study, a combination of pharmacological and molecular tools was utilized to investigate the functional role of tick hemocytes in rickettsiae infection. In *Amblyomma maculatum,* four groups of distinct morphological types of hemocytes were classified according to their defined cytoplasmic projections, presence of intracytoplasmic granules, and the nuclear-cytoplasmic ratio. *In-vivo* bead phagocytosis led to identifying phagocytic and non-phagocytic populations of hemocytes. Clodronate liposome was used to deplete phagocytic hemocytes and elucidate their role in immune response and survival against *Staphylococcus aureus, Escherichia coli*, and *Rickettsia parkeri* infection. Finally, we conducted a comprehensive mRNA profiling study of *Am. maculatum* hemocytes with and without *R. parkeri* infection. Bulk RNA sequencing of hemocytes with and without *R. parkeri* infection revealed a total of 39,249 mRNA transcripts, with 11,301 identified as immune-related. Using a reverse genetic approach (RNAi), we functionally characterized two differentially expressed marker genes in hemocyte phagocytosis. Together, these results represent a significant advancement in our understanding of the basic biology of tick hemocytes and their role in the tick immune response.

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The Long non-coding RNA landscape of host cells during *Coxiella burnetii* infection

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Coxiella burnetii (Cb) is an obligate intracellular respiratory pathogen that grows within host monocytes and macrophages and causes Q fever. Cb replicates inside the *Coxiella* containing vacuole (CCV) and promotes its intracellular growth by modulating innate immune responses through poorly characterized mechanisms. Eukaryotic long non-coding RNAs (lncRNAs) are a regulatory class of transcripts of length > 200 nucleotides that are known to modulate innate immune signaling responses in auto-immune diseases and cancer. Their role however remains largely uncharacterized especially in regulation of host responses during Cb infection. In this study, we aimed to identify LncRNA transcriptome during Cb infection *in vitro*. We conducted a high-throughput RNA-seq analysis of differentiated THP-1 cell lines infected with NMII compared to mock at 1 hour of infection. 1692 genes were significantly altered in this study. STAR alignment, GENCODE and lncFinder databases were employed to identify 161 differentially expressed lncRNAs. These lncRNAs were functionally annotated based on their co- expression analysis with associated coding genes. 55 pairs of lncRNAs and target protein coding genes were identified that functionally enriched for categories like metabolism, endocytic trafficking, transcription, apoptosis, DNA damage and immune response. Amongst these, we selected pairs; lnc-DKK2, DKK2 that enriched for Wnt signaling and lnc-CYP1B1, CYP1B1 for ROS meditated inflammatory response for further analysis. Conservation pattern analysis identified their orthologs in mice. They exhibited hallmarks of regulatory molecules such as spatio- temporal specific expression across 14 days of infection with NMI. *In vitro* infection assay with NMII also showed differential expression profile of lnc-DKK2, DKK2 and lnc-CYP1B1, CYP1B1 at different time points, which indicates a possible involvement of these lncRNAs and associated genes with Cb infection dynamics. In this study, our transcriptome analysis identifies critical lncRNAs modulating host signaling pathways which will be further explored for their mechanisms in Cb pathogenesis.

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Coxiella burnetii as an expression vector for SARS-CoV-2 spike protein: A tool for the study of host-pathogen interactions

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Coxiella burnetii is a Gram-negative obligate intracellular pathogen that causes Q fever, a highly variable disease that typically manifests as an acute flu-like illness but can also result in chronic Q fever or post-acute Q fever chronic fatigue syndrome. While *C. burnetii* whole cell vaccination (WCV) induces potent protection against Q fever, there is limited data indicating that *C. burnetii* vaccination may elicit non-specific immune protection. Prior to studying this *in vivo*, we aim to expand the repertoire of experimental tools to study C. *burnetii*-host immune interactions, knowledge of which is currently lacking. Specifically, the Nine Mile II (NMII) strain of *C. burnetii* was genetically engineered to constitutively express the S1 subunit of the SARS-CoV-2 spike protein on an outer membrane protein, CBU0307, with the aim of using it as a control for studies of host-pathogen immune interactions. The S1 subunit, alone, is sufficient to elicit SARS-CoV-2 immunogenicity as it contains the receptor binding (RBD) used by the virus to enter human cells via interaction with ACE2 receptors. Expression of the S1 subunit on CBU0307 was driven by p1169, a constitutively active *C. burnetii* promotor and directed to the outer membrane protein using the CBU0307 signal sequence. *C. burnetii* S1 expression was confirmed first via immunoblot of both whole cell lysates and outer membrane fractions with SARS-CoV-2 spike and RBD polyclonal antibodies. Further confirmation of expression was observed via immuno-electron microscopy. While immunogenicity is yet to be confirmed, these data mark the first report of non-native protein expression on *C. burnetii*, to our knowledge. If immunogenicity is confirmed, this tool will not only aid in our future investigation of the potential non-specific immune effects of *C. burnetii* vaccination, but it will also allow us to evaluate the feasibility of *C. burnetii* as a vaccine vector.

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Ehrlichia chaffeensis TRP120 targets nuclear innate immune regulator hnRNPA2B1 for degredation

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Ehrlichia chaffeensis (*E. chaffeensis*) is an obligately intracellular bacterium that replicates in mononuclear phagocytes by secreting an array of effectors, including tandem repeat protein 120 (TRP120). TRP120 is a nucleomodulin and has multiple functions in the host nucleus, including ubiquitin ligase activity, which mediates ubiquitination and proteasomal degradation of nuclear tumor suppressor FBW7, thereby increasing oncoprotein stability. Previous studies in our laboratory have shown that TRP120 interacts with heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1), an innate immune response sensor and regulator. Recent investigation of hnRNPA2B1 antiviral activity revealed it promotes the nucleocytoplasmic export of *cGAS*, *IFI16*, and *STING* mRNAs to amplify cytoplasmic innate response signaling to activate type I interferon (IFN) production. These observations led us to hypothesize that *E. chaffeensis* TRP120 targets nuclear hnRNPA2B1 for degradation to destabilize *cGAS*, *IFI16*, and *STING* mRNAs, thereby downregulating innate immune responses to promote intracellular survival. We examined the role of hnRNPA2B1 by siRNA knockdown and observed increased ehrlichial infection. We then examined hnRNPA2B1 nuclear levels by immunofluorescence using confocal microscopy and Western immunoblot and determined that hnRNPA2B1 is degraded during infection. The expression of *cGAS*, *IFI16*, and *IFNB* mRNAs was decreased following siRNA of hnRNPA2B1. Collectively, this study suggests that TRP120 targets hnRNPA2B1 for degradation to destabilize innate immune regulatory mRNAs to evade the innate immune response. Further, this study illuminates the importance of ehrlichial ubiquitin ligases and the ubiquitin-proteasome system for *E. chaffeensis* infection, thereby creating a model for investigating the role of innate immune regulators during intracellular infection by bacterial pathogens.

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Rab27 in tick extracellular biogenesis and infection

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The blacklegged tick, *Ixodes scapularis*, is the most medically relevant arthropod vector of infectious disease in the United States. It transmits over six pathogens of public health relevance, including the emerging human pathogen *Anaplasma phagocytophilum*, but remains critically understudied. *I. scapularis* releases a variety of anti-inflammatory, anesthetic, and anti-coagulant molecules through its saliva during a blood meal. Recently, it was found that extracellular vesicles (EVs) are secreted in its saliva and may impact microbial transmission to the mammalian host. EV biogenesis and function have been studied in mammalian systems, but their role in arthropods of public health relevance remains elusive. In mammals, the large Rab GTPase family is closely associated with EV biogenesis and many of these proteins appear in the *I. scapularis* genome. Here, we report that Rab27 is critical for tick EV biogenesis and infection. Through an *in vivo* model, we have shown that silencing Rab27 impacts tick fitness whether feeding from a naïve mouse or a mouse infected with *A. phagocytophilum*. Furthermore, the tick acquires less *A. phagocytophilum* after Rab27 silencing. *In vitro* experiments show that silencing of Rab27 causes a size increase of tick EVs which indicates that Rab27 is needed to regulate vesicle biogenesis. Overall, we have shown that Rab27 plays an important role in EV biogenesis and the tripartite interactions between vectors, hosts, and microbes.

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Ehrlichia SLiM ligand mimetic activates Hedgehog signaling to engage a BCL-2 anti-apoptotic cellular program

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E. chaffeensis (*E. ch.*) is a gram-negative intracellular bacterium responsible for the emerging zoonotic disease, human monocytic ehrlichiosis (HME). HME is the most prevalent and life-threatening tick-borne disease in the United States. In the past two decades, the incidence of HME has increased 10-fold; however, the number of reported cases is underestimated by 100-fold due to underreporting and underdiagnosing. *E. ch.* hijacks mononuclear phagocytes to escape host defenses through mechanisms executed by tandem repeat protein (TRP) effectors secreted by the type 1 secretion system. In the past decade, tandem repeat protein TRP120 has emerged as a model moonlighting effector. Remarkably, TRP120 has been defined as a nucleomodulin, ligand mimic and ubiquitin ligase. Molecular mimicry is a survival strategy utilized by an array of pathogens to facilitate host-pathogen interactions and exploit host machinery. Recent publications from our laboratory demonstrate that TRP120 is a ligand mimic that directly interacts with various receptors to reprogram the host cell by activating the Wnt and Notch signaling pathways via a novel tandem repeat short linear motif (SLiM). Interestingly, new data demonstrates that TRP120 contains a SLiM to mediate the Hedgehog signaling pathway during infection. Our laboratory discovered homology between TRP120 and Sonic hedgehog, making TRP120 a potential Sonic hedgehog ligand mimic. Data from our laboratory demonstrates the activation of Hedgehog transcription factor, GLI-1 during infection and TRP120 treatment, which is regulated by endogenous ligand Sonic hedgehog. Further, studies demonstrate that *E. ch.* regulates apoptotic genes targeted by Hedgehog signaling, including *BCL-2*. In summary, we determined that *E. ch.* TRP120 modulates Hedgehog signaling via a tandem repeat SLiM to upregulate BCL-2 mediated inhibition of apoptosis.

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Mechanisms of *Coxiella burnetii* whole cell vaccine reactogenicity

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Coxiella burnetii is the causative agent of Q fever. The only approved vaccine for humans, Q-Vax, is not licensed in the United States due to the high rate of local and systemic reactions in previously sensitized individuals. A greater understanding of the immunological mechanisms responsible for these reactions is needed to produce safe and effective vaccines. We previously investigated the immunopathogenesis of Cb WCV reactions by characterizing the infiltrating immune cells in a sensitized mouse model. Immune cells extracted from vaccine site reactions showed an influx of CD4+ and CD8+ T cells and CD4+ T cells were IFNγ+ and IL17a+, indicating a Th1-mediated hypersensitivity reaction. However, the roles of these infiltrating T cells and cytokines in producing these local reactive lesions is still uncertain. Here, we evaluated the roles of CD4+ and CD8+ T cells using antibody-mediated depletion and adoptive transfer experiments. Additionally, we show that injection of whole cell vaccine material derived from Cb leads to chronic persistence of antigen within the injection site which is likely contributing to the granulomatous component of local vaccine reactions. Understanding the mechanisms behind vaccine reactogenicity provide information essential for the development safe and effective novel vaccines against Cb and other pathogens.

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Host targeted drugs as an alternative control of *Rickettsia* infection

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Rickettsia are arthropod-borne intracellular bacterial pathogens. Current protocol for *Rickettsia* treatment is early administration of doxycycline with incomplete efficacy if administered significantly after onset of symptoms. In addition, other antibiotic-based therapeutic approaches are challenging, as these bacteria are intrinsically resistant to the vast majority of available antibiotics. To advance future therapeutic interventions we have undertaken an alternative strategy. As *Rickettsia* are dependent entirely on their host cells for multiple pathogenic functions, we have turned our attention to drugs that modify host cell homeostasis as possible therapeutics (Host-Targeted Therapeutics) with the overall hypothesis that chemical modulation of host functions may create an inhospitable environment for *Rickettsia* growth. Preliminary screening has shown that two major drug classes appear to have relevant anti-*Rickettsia* properties: blood-pressure regulating calcium channel blockers and antipsychotic dopamine-receptor antagonists. Within cell-culture models, we were able to determine drug concentrations that effectively inhibit *Rickettsia* proliferation but are not directly cytotoxic to the host cells. Effective drug concentrations were defined as inhibiting *Rickettsia* proliferation as measured by a fluorescence-based assay without toxicity to host cells as measured by MTT assay. Therapeutic windows were determined by overlaying normalized graphs generated from immunofluorescence and MTT assays. Assessment of host-targeted drugs as anti-*Rickettsia* agents *in vivo* is ongoing. Within mouse models dosing was informed by therapeutic windows derived previously and existing literature used in the initial development of these drugs. Prophylactic administration of host-targeted drugs was compared to treatment with mock and doxycycline treatment in mice challenged with *Rickettsia conorii*. Current data show promising outcomes for Host Targeted therapeutics in murine models of *Rickettsia* infection.

Identification of differentially expressed genes in *Ixodes scapularis* cells involved in vector-pathogen interactions during early *Anaplasma phagocytophilum* infection

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The field of vector-borne disease has progressed from the notion of arthropod vectors as crawling/flying syringes for pathogen delivery, and there is now an improved understanding that the interactions between arthropods and the microbes they host are an important component determining vector competence. One major contributor to the ability of pathogens to colonize vectors is the arthropod's innate immune system. The tick immune system consists of multiple interconnected defense pathways that are expected to act in a coordinated fashion to prevent infection and limit colonization by tick-borne microorganisms. In response, multiple mechanisms are used by pathogens to evade vector immune recognition and destruction. The tick-borne bacterium *Anaplasma phagocytophilum* (*Ap*) employs various strategies to colonize the tick, including inhibiting apoptosis, reducing reactive oxygen species (ROS) production, rearranging the cytoskeleton, and inducing changes in metabolic pathways. However, the responses of the tick during the very earliest stages of infection when the pathogen makes contact with and invades tick cells are not well characterized. Therefore, this study focused on early infection in order to identify proteins involved in pathogen-tick interactions that could provide insight into the early innate immune response of ticks. Using RNAseq, gene expression was compared in uninfected and *Ap*-challenged *Ixodes scapularis* ISE6 cells at timepoints from 5m to 48h post infection. No significant changes in gene expression were observed until 6h, and the greatest number of differentially expressed genes (DEGs) were observed at 24h and 48h. Transcripts for membrane-associated proteins were overrepresented amongst DEGs. Among immunity-associated transcripts, those involved in apoptosis, Toll pathway, and antimicrobial peptides were downregulated, whereas those associated with ROS generation and bacterial binding were upregulated. Upregulated tick genes will be targeted by RNAi knockdown to assess their effect on *Ap* invasion of tick cells and identify tick proteins important for responding to or facilitating *Ap* infection.

Classical complement activation, IgM, and anaphylatoxins shape the immune response to *Rickettsia* infection

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Pathogenic *Rickettsia* reside almost exclusively within a host cell, yet previous studies have demonstrated that the complement system is both activated and essential for the immune response to infection. Defining why a primarily extracellular immune sensory system is essential for control of obligate intracellular bacteria will be vital for our understanding of complement functionality and control of intracellular bacterial pathogens. To further define this indispensable component of the innate immune system, we sought to 1) define the mechanism (s) of complement activation during *Rickettsia* infection and 2) analyze the contribution of complement proinflammatory byproducts to macrophage restriction of *Rickettsia* proliferation. Depletion of specific complement components *in vitro* and *in vivo* demonstrates that both C1q and pre-existing IgM contribute to complement activation; thus implicating both the classical complement system and resident IgM in *Rickettsia*-mediated complement immunosurveillance. A major result of complement activation is production of proinflammatory anaphylatoxins (C3a, C5a, C4a). Through analysis of *Rickettsia* growth in diverse innate immune cells, we demonstrate that anaphylatoxin receptor engagement ultimately produces a cytoplasmic environment that is less conducive to *Rickettsia* growth. Together, these studies highlight the role of the classical complement pathway in innate immunity against intracellular bacteria and implicates resident *Rickettsia*-responsive IgM in the innate response to infection. Additionally, complement anaphylatoxins alter macrophage functionality to control growth of *Rickettsia.* Overall, this data demonstrates that the complement system contributes to immune clearance of *Rickettsia*.

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Functional characterization of a cat flea salivary antigen

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The cat flea (*Ctenocephalides felis*) is an important hematophagous ectoparasite in veterinary and public health by causing direct damage to the host and transmitting various pathogens, including *Rickettsia felis*, the etiological agent of flea-borne spotted fever. The cat flea's salivary components play a crucial role in countering the vertebrate host physiology and immune defense for successful ingestion from its blood. Although several salivary factors have been identified, only a few of them have been functionally characterized. To elucidate the function of the cat flea salivary antigen 1 (also known as transcript *Cf-169*), we applied RNA interference. In the present study, adult cat fleas were pre-fed bovine blood for 72 hours and then fed twice *Cf-169* siRNA (treatment group) or negative scrambled siRNA (control group) for 16 hours. After the initial treatment, salivary glands were dissected 4- and 7-days post exposure (dpe), and the extracted RNA was subjected to reverse-transcriptase quantitative PCR. Gene expression analysis showed a significant decrease in the *Cf-169* transcript levels both at 4 and 7 dpe, confirming the effective suppression of the salivary antigen 1. Additionally, a visible phenotypic change in the treated cat flea feeding behavior and a substantial reduction in excretion at 7 dpe compared to controls was observed. The results showed that salivary glands could take up siRNA from the hemolymph when the molecules were administered through feeding to obtain reliable gene silencing. Further, RNA interference in combination with infection assays can reveal flea-derived genes associated with pathogen infection in the salivary glands and subsequent horizontal transmission. The role of the salivary antigen 1 in *R. felis* transmission is under current study.

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Characterization of the HGE14 family of nuclear-localized T4SS Effectors of *Anaplasma phagocytophilum*

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Anaplasma phagocytophilum (Ap) is a tick-transmitted obligate intracellular zoonotic pathogen that encodes a Type IV Secretion System (T4SS) capable of injecting bacterial proteins, termed effectors, from the infection vacuole into the host cell cytoplasm. Effectors modify cellular processes to favor survival and replication of the bacteria. To better understand this pathogen, our goal is to identify and characterize its effectors. We have characterized a family of effector proteins that are unique to Ap: the HGE14 family in Ap strains HZ and HGE1 contains 10 genes although it appears that only eight are functional. Transcript analysis showed that all paralogs were expressed in HL-60 cells with one consistently more highly expressed than the others. Using a heterologous T4SS translocation assay, it was demonstrated that three paralogs were translocated in a T4SS dependent manner. Six of the proteins have a predicted C-terminal nuclear localization signal (NLS) and localize to the host cell nucleus when ectopically expressed. Deletion of the 11 C-terminal amino acids eliminated nuclear-specific localization as well as T4SS translocation. An antibody raised against one of the HGE14 proteins recognized three of the six nuclear localized paralogs based on Western blot analysis of cells transfected with the individual paralogs. Immunostaining and imaging of Ap-infected cells confirmed that one or more of the effector proteins was expressed and trafficked to the nucleus of the host cell. Western blot analysis shows the paralog with the highest transcript level is also the most abundant at the protein level. Functional characterization of these effectors will inform their role in Ap replication, pathogenesis, and transmission.

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Efficacy evaluation of *Anaplasma centrale* Msp2 hypervariable regions for protection from bovine anaplasmosis

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Bovine Anaplasmosis, caused by *Anaplasma marginale* (Am), is the most prevalent tick-transmitted pathogen of livestock globally. In many parts of the world, a related organism, *Anaplasma centrale* (Ac) is used as a live, blood-borne vaccine, as it causes no or only a mild clinical disease. Ac does not prevent infection with Am, but does prevent acute disease. Ac is prohibited from being used in the US due to the risk of transmitting emerging pathogens. Both of these organisms encode proteins known as Major Surface Protein 2 (Msp2), which is the most immunodominant protein for the organism. Both organisms persist in their host by evading clearance, ie the adaptive immune response by recombining the hypervariable region (HVR) of Msp2 with pseudogene alleles. The study goal was to test whether the Msp2 HVRs encoded by *Ac* are a sufficient source of immune stimulation to provide the clinical protection exhibited by the blood-borne vaccine. Calves were inoculated with recombinantly expressed Ac HVRs. Control groups were inoculated with saponin or infected with the *Ac* live vaccine and compared with the test group. Clinical parameters of packed cell volume (PCV) and percent-parasitized erythrocytes (PPE) were measured following tick challenge with the *Am* St. Maries strain. Western blot analysis demonstrated that the HVR immunizations and Ac live vaccine stimulated an immune response. All animals in the study became infected upon tick challenge. The saponin immunized control group had high PPE's (5.4%) and larger drops in PCV's (14.6%). As expected, the *Ac* immunized animals were protected from acute disease with lower (0.6%) parasitemia and lower drops in PCV (8.6%). The HVR immunized group had intermediate results that were not statistically significant different from either the negative or positive controls. This suggests that the HVR immunogen does not fully recapitulate the protective capacity of the live vaccine.

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Investigating mechanisms by which spotted fever group (SFG) Rickettsiae induce microvascular endothelial cell barrier permeability

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SFG rickettsioses occur worldwide and can be severe. SFG rickettsiae primarily infect endothelial cells leading to increased microvascular endothelial cell (MEC) vascular permeability (VP). Increased VP is the major pathophysiological effect of SFG rickettsioses, but its mechanism is unclear; both rickettsial and host cytokine effects are demonstrated. MEC barrier permeability is linked to changes in intracellular calcium concentrations $[Ca^{2+}]_i$. Using *Rickettsia parkeri* (*RP*)-infected MEC *in vitro*, we showed increased MEC $[Ca^{2+}]_i$, and that calcium chelators (DP-b99, DP-460) and the calcium channel blocker benidipine (but not nefidipine) abrogate *RP*-induced barrier permeability. Thus, we hypothesize that SFG rickettsiae modulate MEC calcium transport systems leading to barrier dysfunction before direct rickettsial damage. To further investigate if *in vivo* regulation of $[Ca^{2+}]_i$ attenuates rickettsia-induced VP, *RP*-infected C3H/HeN mice were injected IP daily with 3 or 10mg/Kg of benidipine, a voltage-dependent T-type calcium channel blocker. Bacterial load (qPCR), histopathology (H&E), and VP (dextran-FITC leakage) were assessed in tissues on d3 and d6 post-infection. Bacterial loads in the spleen, liver, lungs, and brain were increased approximately 10-fold ($p<0.05$) in *RP*-infected mice on d3 post-infection with 10mg/Kg benidipine and 10 to 100-fold ($p<0.05$) on d6 post-infection with 3mg/Kg benidipine compared to the vehicle-treated controls. Increased histopathologic injury and VP was associated with increased bacterial load; however, *RP*-infected mice with 3mg/Kg benidipine on d3 post-infection had reduced VP but similar bacterial load and histopathologic injury as the vehicle-treated mice. Notably, *RP*-infected mice with 10mg/Kg benidipine had accelerated lethality or were euthanized by d3. Although benidipine adversely affected innate immune control of *RP*, reduced VP *in vivo* with 3mg/Kg was still observed, as *in vitro*. This suggests that rickettsia-induced MEC VP involves specific calcium channels that could inform rickettsia-specific VP pathogenesis and help to define precision approaches that do not affect innate immune control of infection.

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Targeted mutagenesis in *Anaplasma phagocytophilum* for modified live vaccine development

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Anaplasma phagocytophilum is an obligate tick-borne bacterial pathogen affecting a wide range of hosts. It is known to infect humans, dogs, cats, cattle, sheep, and horses. To date, no vaccines are available to prevent *A. phagocytophilum* infections and doxycycline/tetracycline administration is the only preferred treatment option. A phage head-to-tail connector protein
(*PHTCP*) gene is identified as essential for *Ehrlichia chaffeensis* and *Anaplasma marginale.* Our recent studies also demonstrated that functional disruption mutation of this gene causes attenuated growth and clearance of the pathogens from vertebrate hosts and offer protective immunity against virulent infection challenges. As *PHTCP* gene homologs are conserved in related rickettsiales, we considered it as the ideal target for both establishing mutagenesis methods for *A. phagocytophilum* and to investigate its value as a vaccine. Molecular methods were employed to generate homologous recombination construct to disrupt the gene. Subsequently, allelic exchange mutagenesis protocol was employed in generating targeted mutation in *A. phagocytophilum*. Mutagenesis was performed on cell-free bacteria recovered from ISE6 tick cell line and cultured in the presence of selection media containing gentamycin and monitored for mCherry expression, as the mutant is expected to contain gentamycin resistance and mCherry protein genes. Clonally purified *A. phagocytophilum* mutant was generated and the presence of functional gene disruption mutation was confirmed by molecular methods in cultured bacteria resisting to gentamycin and expressing mCherry.
This study represents the first development of targeted mutagenesis in *A. phagocytophilum*. Work is now in progress to test its effectivity as a vaccine candidate.
(This work was supported by the PHS grant #s AI152418 and AI070908 from the NIAID, NIH, USA.)

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RNA analysis of Ehrlichia chaffeensis 7 genes spanning from ECH_0659 to ECH_0665 encoding for several phage related proteins and their orthologs in related Anaplasma and Ehrlichia species

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Tick borne anaplasmosis and ehrlichiosis are major health threats to people, companion and agricultural animals. Currently there are no vaccines available to prevent the diseases. Our previous study revealed that the phage head-to-tail connector protein (PHTCP) gene (gene tag # ECH_0660) is essential for bacterial survival, and its disruption aids in the vaccine development. Genes spanning upstream and downstream to ECH_0660; ECH_0659 to ECH_0665 (the 7-gene segment) encode for several phage proteins in *E. chaffeensis.* These genes are conserved in related *Anaplasmataceae* family bacteria. We performed RNA analysis targeting the 7-genes in *E. chaffeensis* and their orthologs in *Ehrlichia canis*, *Anaplasma phagocytophilium* and *Anaplasma marginale* to define the relatedness and impact of ECH_0660 mutations. The analysis was also performed for *E. chaffeensis* ECH_0660 mutant. While, all 7 genes were transcribed in wild-type *E. chaffeensis* Arkansas isolate, ECH_0662 and ECH_0664 gene transcripts were absent for St. Vincent, Heartland and Wakula isolates. Transcription for *E. canis* Jake isolate ortholog for ECH_0662 expression was absent, whereas *A. phagocytophilum* HGE2 isolate and *A. marginale* St. Maries isolate had transcripts for all shared orthologs. We developed targeted mutations also in the orthologs of ECH_0660 for the three pathogens; *A. marginale, A. phagocytophilium,*and *E. canis*, in addition to *E. chaffeensis.* All four species mutants did not produce transcripts for the ECH_0660 orthologs, while mutations having no impact the gene expression from other shared gene orthologs. Defining the RNA expression in mutant bacteria is critical to ensure that there are no off target effects in the gene expression near the mutational sites.

(This work was supported by the PHS grant #s AI152418 and AI070908 from the NIAID, NIH, USA)

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The *A. phagocytophilum* T4SS tick effector contains EPIYA motifs and directs tyrosine phosphorylation

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The bacterium *Anaplasma phagocytophilum* is the second most prevalent tick-borne pathogen in North America and the causative agent of human granulocytic anaplasmosis. Transmitted by the tick, *Ixodes scapularis*, disease is characterized by fever, muscle aches, and nausea which can progress to respiratory distress and organ failure. Upon transmission from the tick vector to the mammalian host, *A. phagocytophilum* encounters a variety of selective pressure as it adapts to new tissue and cell types. This challenge is especially pronounced for *A. phagocytophilum* as an obligate intracellular bacterium. A central virulence mechanism for *A. phagocytophilum* is the Type IV Secretion System (T4SS) which delivers effector molecules into the host cell cytosol. To date, five *A. phagocytophilum* T4SS translocated effectors have been characterized but only in the context of mammalian cells. The molecular interactions and targets of T4SS effectors within tick cells is entirely unknown. Recently we identified the *A. phagocytophilum* effector protein, HGE1_2492, as important for growth and survival within tick cells where it associates with the actin cytoskeleton and alters actin morphology. HGE1_2492 contains two intrinsically disordered novel tandem repeat regions. The C-terminal repeat region is sufficient for association with actin, while the central repeat region is necessary for altering host cell actin morphology. In this central repeat region, we find variants of the bacterial effector motif EPIYA (EPLYA). EPIYA motifs are sites of tyrosine phosphorylation in other effectors and mediate pathogen-host interactions. By fluorescent imaging using an anti-tyrosine phosphate antibody we demonstrated that phosphorylated tyrosine(s) are concentrated at sites of HGE1_2492 localization, dependent on the central EPLYA containing repeat region. HGE1_2492 is the second *A. phagocytophilum* T4SS effector with an EPIYA motif, and the first that appears to be tick specific. Understanding how this pathogen utilizes these effectors to establish infection in different host niches could provide opportunities for interventions.

Drosophila Sting mediates *Coxiella burnetii* infection by regulating oxidative stress

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The Gram-negative bacterium *Coxiella burnetii* is the causative agent of Query (Q) fever in humans and coxiellosis in livestock. *C. burnetii* infects a variety of cell types, tissues, and animal species including mammals and insects, but gaps in knowledge remain regarding the molecular mechanisms that control infection, especially in different genetic backgrounds. Human STING (STimulator of Interferon Genes) induces an innate immune response through the induction of type I interferon (IFN), which regulates *C. burnetii* replication. Furthermore, both STING and *C. burnetii* are sensitive to reactive oxygen species (ROS). In *Drosophila melanogaster*, the functional STING ortholog (Sting) is activated during pathogenic infection and initiates an innate immune response through the NFkB ortholog Relish to induce antimicrobial peptides. Here, we show that Sting-null flies exhibit increased mortality and reduced induction of antimicrobial peptides following *C. burnetii* infection compared to control flies. Sting-null flies induce lower levels of *catalase*, an oxidative stress gene, and generate less ROS, even when infected with a *C. burnetii* strain over-expressing catalase. We find that N-acetyl-cysteine (NAC), and antioxidant, rescues host survival to wildtype *C. burnetii* in the absence of Sting. We are currently examining if NAC restores oxidative stress homeostasis in the absence of Sting, providing a mechanism for protection against *C. burnetii* infection in these mutants. Together, these results establish a role for Sting during *C. burnetii* infection and ROS homeostasis in *D. melanogaster*.

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An investigation of relationship between *Wolbachia* endosymbionts and *Rickettsia felis* in the cat fleas, *Ctenocephalides felis*

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Rickettsia felis is an emerging insect-borne rickettsial pathogen and the causative agent of flea-borne spotted fever. The primary vector and reservoir of this pathogen is cat fleas, *Ctenocephalides felis*. Most transmission cycles of *Rickettsia* include vertical passage in their arthropod hosts as well as transmission to new vectors through the infectious blood of vertebrate amplifying hosts. However, vertical transmission of *R. felis* to cat flea progeny has been highly variable in a laboratory setting, possibly due to the presence of vertically maintained endosymbionts within the vector population. For example, vertically transmitted bacterial symbionts such as *Wolbachia* can influence transmission of pathogens in insects. Thus, it is hypothesized that if *Wolbachia* endosymbionts compete with rickettsiae for stable maintenance in the vector, then the presence of *Wolbachia* will influence the vertical transmission of *R. felis* in the cat fleas. To assess this interaction, we developed a bioassay to knockdown levels of *Wolbachia* in fleas via administration of tetracycline into the blood meal and subsequently assessed flea vertical transmission efficiency of *R. felis* compared to untreated fleas. Results show that both the prevalence and mean rickettsial load of *R. felis* in exposed F_1 fleas was significant higher in antibiotic-treated cohorts, compared to F_1 untreated cohort exposed to *R. felis.* Results also indicate that while *R. felis* infection persisted over the course of feeding in exposed fleas (untreated and antibiotic-treated), vertical transmission of *R. felis* was only observed in the antibiotic-treated flea cohort. The data suggest that antibiotic treatment of parental fleas results in enhanced transovarial transmission of *R. felis* by F_2 fleas. The implication is that disruption of cat flea microbiome facilitates vertical transmission of a rickettsial pathogen. Further, the interaction of endosymbionts is important in understanding the ecoepidemiology of *Rickettsia* in vectors.

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The use of the antigenically variable Major Surface Protein 2 (Msp2) in the establishment of superinfection during natural tick transmission of *Anaplasma marginale* in southern Ghana

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Many vector-borne pathogens have a high degree of genetic variation and thus are able to establish persistent infection and superinfection in the mammalian host, despite the existence of an adaptive immune response. The ability to establish superinfection results in a larger population of susceptible hosts even with high pathogen prevalence. How the genetic variation in these pathogens is used to establish superinfection remains poorly understood. *Anaplasma marginale*, an obligate intracellular, tick-borne, bacterial pathogen of cattle, establishes persistent infection by variation in major surface protein 2 (Msp2), which is composed of approximately six pseudogenes that recombine into a single expression site to produce immune escape variants. Greater than 95% of cattle infected with *A. marginale* harbour multiple strains in regions with high transmission. In this study, we determine if genetically distinct *msp2* pseudogenes and their expression are associated with superinfection during natural transmission in southern Ghana. To accomplish this, we sequenced *msp2* pseudogenes and the expressed variants when animals were initially infected with *A. marginale* prior to seroconversion and again when animals were superinfected with an additional strain following seroconversion. At superinfection, there were one to six new *msp2* pseudogenes in each of the five animals. Of all the Msp2

expressed variants in each animal at superinfection, an average of 36% were derived from pseudogenes that were not previously expressed. These immune escape variants were derived from pseudogenes that were present, but not at expressed at the initial infection and the new pseudogenes introduced at superinfection. Of all expressed variants, an average of 32% were derived from the new pseudogenes in three animals, while none of the new pseudogenes were used in the expression site in two of the animals. Thus, superinfection is associated with the introduction of new pseudogenes, but these new pseudogenes are not over-expressed at superinfection.

Determining transmission determinants of an emerging flea-borne rickettsiosis

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Since its recognition in 1994 as the causative agent of human flea-borne spotted fever, *Rickettsia felis,* has been detected worldwide in over 40 different arthropod species. The cat flea, *Ctenocephalides felis*, is a well-described biological vector of *R. felis.* Unique to insect-borne rickettsiae, *R. felis* can employ multiple routes of infection including inoculation of infectious salivary secretions and potentially flea feces into the skin of vertebrate hosts. Yet, little is known of the molecular interactions governing flea infection and subsequent transmission of *R. felis*. While the obligate intracellular nature of rickettsiae has hampered the function of large-scale mutagenesis strategies, studies have shown the efficiency of *mariner*-based transposon systems in Rickettsiales. Thus, this study aimed to utilize *R. felis* genetic mutants in a flea transmission model to elucidate genes involved in the vector. A *Himar1* transposase was used to generate *R. felis* transformants, in which subsequent genome sequencing revealed a transposon insertion near the 3' end of *sca1.* Alterations in *sca1* expression resulted in unique infection phenotypes. While the *R. felis sca1::tn* mutant portrayed enhanced growth kinetics compared to *R. felis* wild-type during *in vitro* culture, rickettsial loads were significantly reduced during flea infection. Thus, the use of a biologically relevant model implicates *sca1* as an essential factor facilitating *R. felis* infection in the flea.

Anaplasma phagocytophilum AipA interacts with CD13 to facilitate host cell invasion

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Human granulocytic anaplasmosis (HGA) is a severe disease for which no vaccine exists and is emerging at an alarming rate. The causative agent is *Anaplasma phagocytophilum*, an obligate intracellular bacterium with a tropism for neutrophils. Invasins are bacterial surface components that interact with specific receptors to facilitate invasion of host cells. AipA is one of three known *A. phagocytophilum* surface proteins that are critical for infection. A yeast two-hybrid screen indicated that AipA potentially interacts with a C-terminal domain of CD13, a multifunctional protein expressed on most leukocytes. CD13 exhibits alanyl aminopeptidase activity and mediates signaling events that lead to receptor-mediated endocytosis and phagocytosis. The AipA-CD13 interaction was confirmed via co-immunoprecipitation for both recombinant and native AipA. Treating human promyelocytic HL-60 cells and neutrophils with CD13 antibody failed to impair *A. phagocytophilum* cellular adhesion, but reduced infection by 38%. Overexpression of CD13 on the surfaces of HEK-293T cells, which otherwise do not express CD13, resulted in a 40% increase in susceptibility to *A. phagocytophilum* infection. Inhibiting CD13 catalytic activity had no effect on bacterial binding to and entering host cells. However, treating host cells with mAb 452, which crosslinks CD13 to activate Src and downstream signaling that leads to cytoskeletal rearrangement, rescues the ability of AipA antibody-treated *A. phagocytophilum* to infect. Thus, AipA crosslinks CD13 to invoke signaling that promotes infection. Consistent with the fact that CD13-invoked cytoskeletal rearrangement also involves Syk, $AipA_{9-21}$ binding to CD13 elicits Syk phosphorylation. Finally, infection of peripheral blood neutrophils in $CD13^{-/-}$ mice is reduced by 40%, indicating that *A. phagocytophilum* requires CD13 to productively infect neutrophils *in vivo*. These results indicate that *A. phagocytophilum* AipA contributes to the infection process by interacting with CD13 to elicit signaling that culminates in *A. phagocytophilum* cellular invasion.

Types I and II interferon signaling and pathogenesis in the murine model of *A. phagocytophilum* infection

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Anaplasma phagocytophilum (*Aph*) is a tick-transmitted obligate intracellular bacterium that causes human granulocytic anaplasmosis (HGA). Severity ranges from mild to severe hyperinflammatory disease. Plasma IFNg in mouse models peaks after bacterial loads but before maximum pathological injury. *Ifng*$^{-/-}$ mice develop minimal pathology and significantly higher bacterial loads than wild-type, supporting Stat signaling in immune response and pathogenesis. However, *Aph*-infected *Stat1*$^{-/-}$ mice develop severe clinical signs, hyperinflammatory injury, hypercytokinemia, and marked bacterial blood/tissue load increases at d7-14, suggesting an important role for coordinated interferon signaling. Since Stat1 affects type I and II IFN signaling, we hypothesized that activation of both is essential for innate immune control of infection and disease. A129 (*Ifnα/βR*$^{-/-}$), AG129 (*Ifnα/β/γR*$^{-/-}$), and wild type (129Sv) mice were infected to evaluate interferon signaling in disease pathogenesis and pathogen control. AG129 mice demonstrated hyperinflammatory histopathologic tissue damage as described in *Stat1*$^{-/-}$ mice, including loss of splenic white pulp follicular architecture. AG129 mice had between 1-3 log-fold higher bacterial tissue loads than A129 or wild type mice. Infected A129 mice had less severe histopathologic injury than AG129 mice, but more than wild type mice, and also had retained splenic architecture, with spleen and liver bacterial loads not different than wild type. These results demonstrate that type I interferon signaling is dispensable for *Aph* control in early infection, while loss of both type I and II interferon signaling impacts appropriate immune coordination, histopathologic severity, and pathogen control. This provides evidence that balanced type I and II interferon signaling is key for coordinating antimicrobial response. Additionally, since IFNα/β is important for innate immune cell IFNγ production, these data suggest that type I interferons promote effective type II antimicrobial response, including pathogen control, while curtailing hyperinflammatory tissue injury as in severe HGA with macrophage activation syndrome or sepsis.

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The canine host serving as a sentinel species for tick-borne diseases caused by Anaplasma, Ehrlichia, and Borrelia pathogens impacting human health in the USA

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Tick-borne diseases continue to threaten the health of people, dogs and agricultural animals. In the USA, human Lyme caused by *Borrelia burgdorferi* has the highest incidence, followed by diseases resulting from *Ehrlichia* and *Anaplasma* species. We investigated the prevalence of these diseases in dogs and compared with human data as the same pathogens also cause infections in the human host. Clinically suspected dog blood samples from across the USA were assessed for pathogen-specific antibodies. An ELISA assay was performed for *B. burgdorferi*, while indirect immunofluorescence assay was used for *E. chaffeensis, E. canis* and *A. phagocytophilum.* A total of 1,340 samples were assessed for antibody analysis. Two hundred and eighty-six samples tested positive for *A. phagocytophilum* (21.3%). Similarly, 228 samples were positive for *E. chaffeensis* (16.9%), 233 samples positive for *E. canis* (17.3%) and 366 samples being positive for *B. burgdorferi* (27.2%). Some of the *Ehrlichia* positives are likely the result of antigenic cross-reactions between the two *Ehrlichia* species. Similarly, some *A. phagocytophilum* positives may represent *A. platys* positives. Co-infection with both *Anaplasma* and *E. chaffeensis* species was observed in 65 dogs; 64 dogs were positive for both *Anaplasma* and *E. canis*; 76 dogs were double-positive for *Anaplasma* and *Borrelia*; 34 for *Ehrlichia* and *Borrelia*; and 8 dogs tested positive for all three species. We observed a significant overlap in the geographical distribution of the samples that tested positive for the pathogens belonging to all three diseases in dogs. These data are similar to the CDC-reported human prevalence data for tick-borne diseases for anaplasmosis, ehrlichiosis, and borreliosis, thus, suggesting that the occurrence of tick-borne diseases in dogs is very similar to documented human cases. Monitoring canine infections, therefore, has important implications for both human and the companion animal health.

(This work was supported by the Abaxis, Inc., Union City, CA and PHS grant #s AI070908 and AI152418 from the NIAID, NIH, USA.)

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Coxiella burnetii requires type IVB secretion system to suppress hostTLR3/TRIF-dependent NF-KB-activation

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Coxiella burnetii (Cb), the causative agent of Q fever, replicates inside a Coxiella-containing vacuole within monocytes and macrophages modulating NF-κB-dependent host innate immune processes. A *Cb* (RSA439, phase II) growth defect in C57Bl/6 bone marrow-derived macrophages (BMDM) is restored in BMDMs deficient in TLR2, MyD88 or TNFαR, supporting a model for NF- KB-regulation-dependent restrictions during infection. *Cb* requires a functional type IVB secretion system (T4SS) to modulate host NF-KB signaling and T4SS effectors, including Cbu1217 (NopA) and Cbu0513 (CinF) are implicated as mediating these modulatory roles. Yet, characterization of the range of *Cb* T4SS effectors which directly or indirectly modulate NF-KB signaling during infection remains incomplete. To determine at which steps of NF-KB signaling the *Cb* T4SS effectors modulate the host, we tested molecular components both up and down-stream in the NF-KB pathway. We used multiple Toll-like receptor (TLR) ligand-inducing NF-KB activation screens to identify how *Cb* regulates NF-KB activation. TRIF-dependent TLR ligands in THP1 NF- KB reporter cells quantitatively demonstrated that *Cb* utilized T4SS to selectively inhibit host TLR3/ TRIF-induced NF-KB activation. Furthermore, RT-qPCR confirmed that *Cb* used T4SS to suppress the transcription of NF-KB-mediated host TNF-alpha, IL8, and BCL-3 in THP-1 cells induced with Poly IC (a TLR3 agonist). Comparison between *Cb* NMII and 1'DotA (T4SS deletion mutant) in TRIF$^{-/-}$ -THP-1 NF-KB reporter cells demonstrated a loss of TLR3-induced NF-KB activation in cells infected with 1'DotA. Moreover, laser scanning confocal microscopy showed that both TLR3 and TRIF co-localize with *Cb* NMII vacuoles. Collectively, these data demonstrate that during infection, *Cb* blocks TLR3/TRIF-dependent NF-Kb signaling in a T4SS-dependent manner and provides an opportunity to identify specific T4SS effectors which mediate this process.

Tick regulation of epidermal skin immunity facilitates an advantageous arthropod feeding environment

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Hard ticks are hematophagous arthropods of public health importance. Following a tick bite, these arthropods take prolonged, continuous bloodmeals that facilitate pathogen transmission. Successful bloodmeals are attributed to components of the tick saliva that alter inflammation, inhibit hemostasis, and block pain and itch responses in the mammalian skin. Recent studies have reported that extracellular vesicles (EVs) in the saliva enable tick feeding and redirect skin immunity. Here, we demonstrate that EVs promote infection of rickettsial agents *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*, as well as regulate resident epidermal γδ T cells. Epidermal γδ T cells interact with keratinocytes, which comprise about 95% of the skin epidermal layer, to ensure optimal maintenance of tissue homeostasis and epithelial repair. We demonstrate that *Ixodes scapularis* ticks significantly reduce epidermal γδ T cells at the bite site for an optimal feeding environment. Using flow

cytometry, we profiled the epidermal γδ T cell activation status in the presence or absence of EVs. Furthermore, we employed single cell RNA sequencing coupled with animal models devoid of epidermal γδ T cells to evaluate the directionality of the skin immune response during a tick bite. Collectively, this work broadens our knowledge of principles of immunology and vector-host interactions.

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Hemocyte-mediated immune response to a bacterial infection in the cat flea (*Ctenocephalides felis*)

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Although the significance in natural transmission cycles remains unclear, rickettsial pathogens cross the midgut epithelium, migrate through the hemocoel (body cavity), and, in the case of *Rickettsia felis* (flea-borne spotted fever), invade the salivary glands of susceptible flea species. Once inside the flea hemocoel, rickettsial pathogens must evade immune cells called hemocytes. Among invertebrates, hemocytes provide defense against foreign microbes via phagocytosis, cellular encapsulation, and production of humoral immune factors. However, current information on the types of hemocytes fleas produce, their relative abundance, and their functions is limited. This is a significant gap in knowledge, as improved understanding of how fleas defend themselves from systemic infections could lead to the development or refinement of disease control strategies. Toward this end, we investigated the strength of the hemocyte-mediated immune response in cat fleas (*Ctenocephalides felis*) against a model Gram-negative bacterium, *Escherichia coli*. We delivered an *E. coli* infection with a septic pinprick, and measured the following: (1) *in vivo* bacteria killing efficiency; (2) quantification of circulating hemocytes; and (3) the relative capacity of hemocyte phagocytosis. At 24 hours post infection, we found that the bacterial load in the flea hemocoel decreased by 54% compared to the infectious dose originally administered. Additionally, the number of circulating hemocytes changed significantly between naïve, injured (sterile pinprick), and *E. coli*-infected fleas (ANOVA: $P < 0.0001$). Specifically, *E. coli*-infected fleas contain more circulating hemocytes (2209 ± 204 SEM) than naïve (1197± 122 SEM) or injured (1518 ± 109 SEM) fleas. Moreover, our preliminary results suggest that the most abundant flea hemocyte types respond to bacterial challenge by phagocytosis. Overall, this work yields important insight into how fleas interact with Gram-negative bacterial pathogens in their hemocoel, which can directly affect transmission dynamics of flea-borne rickettsiae.

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Anaplasma phagocytophilum recruitment and infection of neutrophils in a 3D model of human dermal microvessels

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Anaplasma phagocytophilum (*Aph*) is an emerging rickettsial pathogen causing human granulocytic anaplasmosis (HGA), a common tick-borne disease in the US. How *Aph* reaches the blood from the tick bite wound is unknown yet important since the skin is the site of first contact between pathogen and host. *Aph*-infected neutrophils are present in the dermis of sheep experimentally infested by infected *I. ricinus* ticks, but how *Aph* enters the vasculature is not understood. We hypothesize that neutrophils are recruited from blood to the tick bite site, become infected with *Aph*, and then intravasate into a dermal microvessel. We established an *in vitro* 3D microvessel model using DAX-1 chips, human dermal microvascular endothelial cells (HDMEC), and "dermal" extracellular type 1 collagen matrix (ECM) to discern key events in *Aph* bloodstream invasion. *Ex vivo* human neutrophils added to the microvessel extravasate by 24h in response to cell-free *Aph* in the ECM 1.8 (mean ± 0.2 SEM) times more often compared to no stimulus ($p<0.01$). In extravasation experiments, small numbers of infected neutrophils were observed in the ECM, verifying *in situ* infection by cell-free mCherry-*Aph*. To demonstrate reverse transmigration, infected and uninfected neutrophils and ATRA-differentiated HL-60 cells (dHL-60) were added to the ECM of TNF-α-stimulated or unstimulated HDMEC. TNF-α stimulation accelerated migration ($p<0.05$) toward the HDMEC barrier for both uninfected neutrophils (2.9±0.1 fold) and uninfected dHL-60 (4.3±0.3 fold) compared to no TNF-α. In contrast, TNF-α had no impact on migration of *Aph*-infected neutrophils or dHL-60 cells. Using this 3D *in vitro* model, we identified 2 of 3 phases required for *Aph* dissemination: i) neutrophil migration to *Aph*; and ii) extravasated neutrophil *Aph* infection within the ECM. The precise mechanism(s) by which infected cells reverse transmigrate is still under investigation, including the impact of other stimuli such as resident immune cells and tick saliva.

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Regulator of actin-based Motility (RoaM) downregulates actin tail formation by *Rickettsia rickettsii* and is negatively selected in mammalian cell culture

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The etiological agent of Rocky Mountain spotted fever, *Rickettsia rickettsii*, is an obligate+ intracellular pathogen that induces the polymerization of actin filaments to propel the bacterium through the cytoplasm and spread to new host cells. Cell-to-cell spread via actin-based motility is considered a key virulence determinant for spotted fever group rickettsiae, as interruption of *sca2*, the gene directly responsible for actin polymerization, has been shown to reduce fever in guinea pigs. However, little is known about how, or if, motility is regulated by the bacterium itself. We isolated a hyper-spreading variant of *R. rickettsii* Sheila Smith that produces actin tails at an increased rate. *A1G_06520* (*roaM* [regulator of actin-based motility]) was identified as a negative regulator of actin tail formation. Disruption of RoaM significantly increased the number of actin tails compared to the wild-type strain but did not increase virulence in guinea pigs; however, overexpression of RoaM dramatically decreased the presence of actin tails and moderated fever response. Localization experiments suggest that RoaM is not secreted, while reverse transcription-quantitative PCR (RT-qPCR) data show that various levels of RoaM do not significantly affect the expression of the known rickettsial actin-regulating proteins *sca2*, *sca4*, and *rickA*. Taken together, the data suggest a previously unrecognized level of regulation of actin-based motility in spotted fever group rickettsiae. Although this gene is intact in many isolates of spotted fever, transitional, and ancestral group *Rickettsia* spp., it is often ablated in highly passaged laboratory strains. Serial passage experiments revealed strong negative selection of *roaM* in Vero 76 cells. The findings also suggest that passage of rickettsial strains in cell culture may cause the bacteria to lose essential genes that are no longer conserved under natural selective pressure. These findings are likely relevant to the interpretation of studies concerning virulence determinants of rickettsiae.

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The CD36 homolog Croquemort binds infection-derived lipids and initiates antibacterial immunity in *Ixodes scapularis*.

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Ixodes scapularis ticks transmit several human pathogens of public health importance, including the rickettsial agent *Anaplasma phagocytophilum* and Lyme disease spirochete *Borrelia burgdorferi*. During a blood meal, *I. scapularis* may acquire bacteria and mount an immune response that limits colonization. Recent studies in these evolutionarily ancient arthropods revealed formerly unknown mechanisms of immune recognition, which may have broader implications for the evolution of innate immunity. Our group previously discovered that lipids derived from *A. phagocytophilum* infection stimulate the tick immune deficiency (IMD) signaling pathway, which restricts colonization of tick-borne bacteria. However, potential receptors for the tick IMD pathway remain elusive due to a lack of homology with *Drosophila* signaling components. Here, we report the tick homolog of Croquemort, a CD36-like lipid scavenger receptor, as a receptor for immunostimulatory lipids. We determined ligand-protein interactions between the ectodomain of Croquemort and the lipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) by pull-down assay, advanced structural modeling, and surface plasmon resonance. Furthermore, we developed a novel ectopic expression technique in *I. scapularis* cell lines to demonstrate the plasma membrane localization of Croquemort. Nymphs silenced for *croquemort* exhibit impaired fitness, an inability to reach full repletion, and delayed molting to adulthood due to deficient ecdysteroid synthesis. Notably, we discovered that Croquemort initiates the IMD and jun N-terminal kinase (JNK) signaling pathways through multiple *in vivo* and *in vitro* assays. We determined that Croquemort relays antimicrobial signals, regulates immune gene expression, and limits acquisition of tick-borne bacteria. Collectively, our findings establish an ancient antibacterial immune response in arthropods and contribute to new scientific paradigms in tick-borne diseases.

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TNF-a-dependent C-type lectin Mincle plays a significant role in innate immune recognition in the brain during scrub typhus infection in both outbred CD-1 and inbred C57BL/6 mice

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Scrub typhus is a disease caused by the bacterium *Orientia tsutsugamushi.* Neural pathologies of scrub typhus include meningoencephalitis, acute disseminated encephalomyelitis, and cerebral venous thrombosis. Despite increased cases of scrub typhus, little is understood about the neural pathologies, including the mechanism of disease manifestation and immunological response. Our lab is interested in host pattern recognition receptors (PRRs), specifically the C-type lectin receptor (CLR) Mincle, and how it modulates innate immune response. This study aims to determine if mincle plays a role in immune recognition in the brain using two different mouse models, C57BL/6 (inbred), and CD-1 (outbred). During infection with the same challenge, CD-1 mice succumb to infection while the C57BL/6 mouse survive. We hypothesize that the TNF-a-dependent CLR Mincle plays a significant role in innate immune recognition in the brain during infection. Additionally, CD-1 mice will have preferentially upregulated mincle mRNA levels compared to C57BL/6 mice. Our previous findings implicate mincle as one of the primary CLRs, regulating the recognition of and subsequent cytokine responses to *O. tsutsugamushi*. *In vitro* studies supported this conclusion; bone marrow-derived macrophages were infected with live or heat-killed bacteria. The CLR and TLR profiles were analyzed along with the proinflammatory state. Our findings showed that macrophages infected with live bacteria had a higher mincle expression than heat killed bacteria, which correlated with the upregulation of proinflammatory genes (*Cxcl9*, *Ccl2*, *Ccl5*, *Nos2*, *IL27*). *In vivo* studies with mincle knockout macrophages showed a significantly reduced proinflammatory state. Since *O. tsutsugamushi* recognition by mincle promotes a proinflammatory state, this could potentially explain severe neural inflammation and immune dysregulation observed in the brain and, if left untreated, could lead to mortality.

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The impact of caspase-8 and STING interactions on extrinsic apoptosis during *Coxiella burnetii* infection

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Coxiella burnetii is an obligate intracellular bacterial pathogen and the causative agent of the global zoonotic disease Q Fever. A greater understanding of how *C. burnetii* interacts with host immune signaling pathways is needed to develop new, targeted therapeutics. Previous research has shown that *C. burnetii* inhibits host cell apoptosis, but bacterial interactions with extrinsic apoptotic signaling, such as with caspase-8, remain largely unknown. Intriguingly, insect homologs of caspase-8 have been shown to interact with the type I IFN regulator STING to promote immune signaling, but whether this interaction or its significance to immunity is conserved in mammals has not been examined. In this research, we investigate interactions between human caspase-8 and STING, as well as how the activity of these proteins impacts *C. burnetii* infection. We hypothesize that STING and caspase-8, both individually and through their interactions, have roles in regulating apoptotic and interferon signaling that are important during *C. burnetii* infection. To evaluate caspase-8 interactions with STING, we examined the co-localization of STING and caspase-8 in human cells using immunofluorescence microscopy, and the impact of STING signaling on caspase-8-mediated apoptosis. Further, we interrogated how this cell death signaling is influenced by *C. burnetii* by western blot and annexin V/PI staining. The results of this study will aid in uncovering the relationships between STING, caspase-8, and *C. burnetii* infection, thereby providing necessary information for effectively targeting these pathways to enhance the immune response against *C. burnetii*.

Ehrlichia chaffeensis activation of notch signaling stabilizes xiap expression to inhibit caspase activation

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We have recently reported that *E. chaffeensis* evasion of host defenses of the macrophage involve activation of conserved host signaling pathways, including Wnt, Hedgehog and Notch signaling through molecular mimicry. TRP120 activates the evolutionarily conserved Notch signaling pathway using molecular mimicry involving a short linear motif (SLiM) Notch ligand mimetic found within the TR domain. Additionally, we have also shown that TRP120 ubiquitin ligase activity results in increased levels of oncoproteins including induced myeloid leukemia cell differentiation protein (MCL1) and Notch intracellular domain (NICD) which are important for regulating cell survival and apoptosis. Therefore, *E. chaffeensis* induced Notch signaling and increased levels of NICD during *E. chaffeensis* infection may play a direct role in inhibiting apoptosis. Activation of Notch assists in inhibition of apoptosis by stabilizing expression of an anti- apoptotic protein, XIAP. Caspases cleave XIAP into two BIR fragments, one of which is a BIR- RING fragment that is a potent inhibitor of intrinsic apoptosis. Thus, we hypothesize that *E. chaffeensis* TRP120 activates Notch signaling to increase Notch intracellular domain (NICD) levels for XIAP stabilization and inhibition of caspase activation. We investigated protein and gene expression levels of XIAP during *E. chaffeensis* infection and demonstrated an increase in XIAP expression. Interestingly, cleavage of XIAP into the BIR3-RING domain occurred during later timepoints of infection suggesting that *E. chaffeensis* activated Notch signaling results in inhibition of intrinsic apoptosis. We also examined caspase levels during *E. chaffeensis* infection and found increased levels of procaspase-3 in *E. chaffeensis*-infected THP-1 cells in comparison to uninfected cells, while increased gene expression of caspases-3. -7 and -9 was demonstrated over the course of infection. These results indicate that TRP120 Notch activation leads to XIAP stability to assist in inhibition of intrinsic apoptosis during *E. chaffeensis*

infection. Overall, this study will identify a novel mechanism by which E. chaffeensis stabilizes XIAP through Notch signaling to increase levels of NICD, thereby preventing caspase activation and apoptosis. Inhibition of apoptosis through Notch signaling provides further evidence that E. chaffeensis hijacks evolutionarily conserved signaling pathways to evade host defense mechanisms for intracellular survival.

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Evaluation of qPCR assay using target gene containing repetitive sequences for *Rickettsia typhi* detection in patients with acute febrile illness

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Murine typhus is a flea-borne disease caused by *Rickettsia typhi* that typically presents as an acute febrile illness. Mortality associated with untreated infection has been estimated as 0.4%. The diagnosis is easily missed, leading to delays in receiving appropriate treatment. A qPCR targeting a single gene for *R. typhi* is widely applied for diagnosis, however, this has low sensitivity for the detection of bacterial DNA in patients' blood. We aimed to optimize qPCR detection by targeting a gene containing repetitive sequences, sca2 (surface cell antigen 2) of *R. typhi.* We compared diagnostic accuracy with the standard assay targeting single sequence ompB (outer membrane protein B). We searched for the region containing the maximum number of repetitive sequences of sca2 for primer and probe design using tandem repeat finder. Primer specificity was tested with several strains of *R. typhi* and other bacteria using *in silico* analysis and PCR. Specificity, sensitivity and bacterial load determination for both assays were compared using stored positive and negative buffy coat samples (n= 54), confirmed by *Rickettsia* culture. Specificity for both assays was 100% (95% CI, 91.80-100) and sensitivity was 81.82% (95% CI, 52.30-96.77). Ten retrospective samples with *R. typhi* detected by IFA (n=10) were tested using both assays. Four samples had detectable sca2 (40%) but not ompB. In the first group of 54 samples, bacterial DNA load determination using sca2 gene was significantly greater than with the ompB gene at approximately 2.933 (median, 16,500 copies/μL; IQR, 13,045 to 40,000 versus median, 19.25 copies/μL; IQR, 11.11-56.62, $P < 0.0001$). This study suggested qPCR targeting sca2 may increase the chance for detection of *R. typhi* in patients with low bacterial DNA concentrations and it may be useful for investigating rickettsia clearance times for antibiotic treatment clinical studies.

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Investigation of *Ehrlichia chaffeensis* TRP120 as a PDGF ligand mimic

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Human monocytic ehrlichiosis (HME) is an emerging, tick-borne zoonosis with a potentially fatal manifestation. Despite a small genome, the causative agent of HME, *Ehrlichia chaffeensis* (*E. ch.*), infects and survives within mononuclear phagocytes largely thanks to the actions of tandem repeat protein 120 (TRP120). Using short linear motifs (SLiMs), TRP120 is capable of mimicking human pro-survival ligands to hijack host cell signaling pathways and prevent apoptosis. SLiMs in TRP120 have been found to share homology with notch, wnt, and hedgehog ligands, and activate these pathways through interactions with their respective receptors. Recently, sequence alignment revealed that TRP120 demonstrates some homology with another human pro-survival, anti-apoptotic ligand: platelet-derived growth factor (PDGF). This finding spurred preliminary investigation into whether TRP120 possesses a novel SLiM mimetic capable of appropriating PDGF signaling to inhibit apoptosis and increase bacterial survival during *E. ch.* infection. THP-1 cells, a human monocytic cell line, were infected with *E. ch.* or treated with a peptide encompassing the TRP120 amino acid sequence homologous with PDGF. Cell samples were collected various timepoints post-treatment, and subjected to immunofluorescent analysis. Antibody staining for the phosphorylated (active) PDGF receptor revealed that PDGF receptor activation in THP-1 cells increased over time during infection with *E. ch.*, and the same result was observed after treatment with TRP120 peptide. Additionally, qPCR analysis demonstrated that mRNA expression of the PDGF receptor significantly increases in THP-1 cells infected with *E. ch.* Taken together, these findings suggest that the *E. ch.* virulence factor TRP120 does function as a PDGF ligand mimic as part of a novel survival mechanism unreported in any other intracellular bacterium.

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Tick hemocytes at the single cell level

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The blacklegged tick *Ixodes scapularis* transmits several pathogens of public health relevance, including *Anaplasma phagocytophilum*. To establish infection in the tick, these microbes must overcome the anti-microbial defenses generated by tick specialized immune cells, or hemocytes. Nevertheless, the full functional diversity, molecular characterization, and cellular differentiation process of tick hemocytes remain elusive. Here, we have successfully profiled the transcriptional signature of 1277 hemocytes from non-engorged ticks using single cell RNA sequencing (scRNA-seq). This genetic characterization revealed five clusters (prohemocytes, granulocytes, plasmatocytes, intermediate-state hemocytes, and an undefined group). We identified markers of each cluster and predicted three different lineages involved in their maturation, placing prohemocytes at the starting point of the hemocyte lineages, followed by intermediate-state hemocytes, which give rise to mature cell types such as plasmatocytes and granulocytes. These findings demonstrate a higher complexity in tick hemocytes compared to previous morphologically defined subtypes. Altogether, our work provides a base for functionally characterizing tick hemocytes upon metabolic alterations or microbial infections and will serve as valuable resource to the vector biology and rickettsial community.

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Drosophila melanogaster as a chassis for understanding bacterial effector targets of arthropods

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Many important arthropod vectors also harbor associated endosymbionts, some of which may modulate infection dynamics with human pathogens. These endosymbionts are difficult to study as they are host associated, often cannot be cultured outside of host cells, and are intractable to genetic modification. Here we present our bioinformatic studies identifying candidate effectors across a large swathe of arthropod-borne endosymbionts and specific studies using the model host *Drosophila melanogaster* to characterize the function of these proteins in the host cellular context. We focus on effectors of *Spiroplasma poulsonii* and *Wolbachia pipientis*. *Spiroplasma poulsonii* is a helical mollicute, distantly related to mycoplasmas, known for their intracellular infection of mammalian epithelial cells. Bacteria of the genus *Spiroplasma* are found in many arthropod lineages and *S. poulsonii* is a maternally transmitted, intracellular parasite of *Drosophila melanogaster* that kills all male offspring. *Wolbachia* are alpha-proteobacteria, part of the anciently intracellular *Anaplasmataceae*, and related to the important human pathogens *Anaplasma*, *Rickettsia* and *Ehrlichia*. However, *Wolbachia* do not infect mammals, but instead are well known for their reproductive manipulations of insect populations. We will show our approach, using transgenic *Drosophila,* and high-throughput screens, to identify host targets of toxins secreted by both symbionts. Using this method, we have identified several genetic and physical interaction between toxins produced by these bacteria and host proteins. Our results pave the way for future work on mechanisms of symbiosis in these enigmatic symbionts.

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Rickettsia species secret effector(s) that modulates intracellular trafficking to establish a replicative niche in host cytosol.

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Intracellular pathogens utilize their effector arsenal to reprogram eukaryotic host cell intracellular trafficking and evade its innate defense responses to establish an intracytosolic niche. For *Rickettsia* species (spp.), Gram-negative obligate intracellular pathogens, the fundamental cellular processes of infection, which include host cell entry via induced phagocytosis, escape from the lysosomal fusion of phagosome/autophagosome into host cytosol, and intercellular spread, remains largely an enigma. Phospholipase A_2 (PLA_2) activity has long been suspected to mediate rickettsial phagosomal escape, as well as entry into and host cells and exit. Recently, we reported that **1)** *R. typhi* Pat1 and Pat2 are surface exposed proteins, which translocated from *R. typhi* into the host cytoplasm, **2)** pretreatment with anti-Pat1 or anti-Pat2 antibodies (Abs) blocked *R. typhi* infection of host cells and also delayed rickettsial phagosome escape, and **3)** yet to be identified is the eukaryotic host cofactors for both Pat1 and Pat2 PLA_2 activities. We hypothesize that specific host molecules spatiotemporally regulate rickettsial phospholipases to facilitate colonization in the metabolite-rich host cytosol for intracellular growth and subsequent intercellular spread. In this effort, we demonstrated that overexpression of *R. typhi* Pat1 (wild type) mostly localized to the plasma membrane (PM) and causes cell rounding. In contrast, an active site (SD) mutant of Pat1, which is localized to PM dese not induce cell rounding. Intriguingly, overexpression of N- or C-terminal Pat1 resulted in a diffused localization pattern and no cell rounding, suggesting that the enzymatic activity of Pat1 is required for its biological function. Furthermore, we determined host protein interactome by ectopically expressing Pat1 into HEK293T cells followed by MS-analysis and have identified 14 putative interacting partners. We prioritized the validation of the identified host target proteins based on their cellular functions that seem relevant to pathogen infection and showed Rab13, which involves in $PI(3,4,5)P_3$-enriched phagocytic cup closure, co-localized with Pat1. Further works are in progress to validate the interaction of host proteins with Pat1 and their biological function to support rickettsial intracytosolic colonization.

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A pleiotropic metabolite affects microbial infection and arthropod vector fitness

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Viewing interspecies relationships through the lens of bioenergetics enables a flexible conceptual framework to understand why virulence is context-dependent in arthropod-borne diseases. Here, we engineered a system of metabolic interdependence in *Ixodes scapularis* where nutrients were allocated according to the glycolytic or oxidative phosphorylation cellular state. The rickettsial agent *Anaplasma phagocytophilum* and the Lyme disease spirochete *Borrelia burgdorferi* induced glycolysis during infection and inhibition of oxidative phosphorylation enhanced microbial colonization of tick cells. Through an unbiased metabolomics approach, we discovered that β-aminoisobutyric acid (BAIBA) was an important metabolite for tick-microbe interactions. Whereas distinct levels of BAIBA affected tick weight and survival *in vivo,* disrupting BAIBA levels through genetic manipulation of catabolic enzymes reduced bacterial infection and restores tick fitness. Collectively, the metabolite BAIBA draws antagonistic pleiotropy on seemingly unrelated evolutionary traits in *Ixodes scapularis* ticks. Bioenergetics and resource allocation have yet to be explored as a strategy to constrain the public health burden of arthropod-borne diseases.

Omics-derived evidence that *Orientia tsutsugamushi* both pirates and modulates host central metabolism during infection

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Orientia tsutsugamushi is a Gram-negative obligate intracellular bacterium that causes scrub typhus in humans. While grossly neglected, scrub typhus is a globally emerging and potentially fatal disease with an estimated one million new cases reported annually in endemic regions. Following infection of physiologically distinct host cells, *O. tsutsugamushi* escapes endosomal processing to reside within its replicative niche, the eukaryotic cytoplasm. The eukaryotic cytoplasm is a nutrient-limited microenvironment. Therefore, the ability of *O. tsutsugamushi* to proliferate within the cytoplasm of physiologically distinct cell types suggests *O. tsutsugamushi* (1) has a substantial metabolic capacity to counter nutrient restrictions; or (2) has evolved to modulate eukaryotic metabolism to pirate host nutrients. For an obligate intracellular bacterium, *O. tsutsugamushi* has a relatively large genome (~2 Mb) due to an abundance of repetitive sequences yet encodes for a limited number of central metabolic genes. Specifically, the *O. tsutsugamushi* chromosome encodes for only three glycolytic enzymes, two pentose phosphate pathway (PPP) enzymes, and the last six enzymes of the TCA cycle. Based on *in silico* predictions alone, to compensate for these metabolic deficiencies *O. tsutsugamushi* must rely on its host to supply precursor metabolites required for intracellular proliferation. Using omics approaches, we interrogated host metabolism to provide experimental evidence that both metabolites and biosynthetic pathways of host central metabolism are modulated during *O. tsutsugamushi* infection. Metabolomics revealed that >80% of significantly altered host metabolites are associated with glycolysis, PPP, TCA cycle, or amino acid metabolism in infected cells. Complementary RNAseq data further implicated host glycolysis as a pathway affected by *O. tsutsugamushi*, as 50% of host glycolytic enzymes were differentially regulated during infection. Taken together, these data provide initial insights into discovering the metabolic intricacies that support *O. tsutsugamushi* intracellular fitness.

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Shaping the understanding of the Unfolded Protein Response and Vector Competency in *Ixodes scapularis*

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Of the U.S. vector-borne disease cases reported to the CDC in 2019, 91% of them were attributable to ticks. *Ixodes scapularis,* the North American deer tick, can transmit up to 7 different pathogens that effect human health including *Borrelia burgdorferi,* the causative agent of Lyme disease, and *Anaplasma phagocytophilum,* the causative agent of human granulocytic anaplasmosis. We have limited knowledge about what factors influence vector competency, which is defined as the ability of an arthropod to harbor and transmit pathogens. Recent work has shown that a vectors' immune system is able to restrict pathogen colonization. However, little is known about the immune system of non-insect arthropods as arthropod immunity has primarily modeled in *Drosophila*. Cellular stress responses, like the unfolded protein response (UPR), are increasingly being linked to innate immunity. Therefore, in this project we asked if the *I. scapularis* UPR cross-talks with innate immune pathways in the tick to limit bacterial colonization. The UPR is a highly conserved mechanism across species and is regulated by three transmembrane receptors, with IRE1α being the most conserved. In mammals, upon activation, IRE1α recruits TRAF2 leading to NF-κB signaling. We first determined an up regulation of key UPR genes in infected *Ixodes*. Using protein modeling and immunoprecipitation, we show that IRE1α and TRAF2 interact in *I. scapularis*. *In vitro* and *in vivo* findings show that both IRE1α and TRAF2 limit bacterial colonization of *A. phagocytophilum* and *B. burgdorferi.* Upon further investigation, activation of IRE1α and TRAF2 signaling lead to the induction of the Immune Deficiency (IMD) pathway's NF-κB-like factor, Relish, indicating a linkage to arthropod immunity. To our knowledge, this is the first time that cellular stress responses have been implicated in influencing vector competency. Overall, by characterizing the interplay between stress responses and immunity, we can understand how ticks regulate acquisition, maintenance, and transmission of tick-borne diseases.

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Investigation of the potential involvement of RIPK3 in *Orientia tsutsugamushi* inhibition of NF-κB

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Orientia tsutsugamushi causes scrub typhus, a potentially fatal and emerging zoonosis. This obligate intracellular bacterium has evolved to counter immune surveillance and defense mechanisms. We previously determined that *O. tsutsugamushi* prevents nuclear accumulation of NF-κB and linked this phenomenon to two ankyrin repeat-containing effectors, Ank1 and Ank6. Both contain N-terminal ankyrin repeat and C-terminal PRANC (pox proteins repeats of ankyrin C-terminal) domains, the latter of which co-opts the SCF ubiquitin ligase complex and is required for NF-κB modulation. This bipartite architecture is shared with members of an orthologous family of *Chordopoxviridae* proteins that also negatively regulate NF-κB. One such ortholog, cowpox CPXV006, additionally binds receptor-interacting serine/threonine-protein kinase 3 (RIPK3) and promotes its SCF-dependent proteasomal degradation using its PRANC domain. RIPK3 is the master activator of necroptosis, a highly inflammatory form of cell death that can involve NF-κB activation. In this study, quantitative assessment of all 19 distinguishable *O. tsutsugamushi* Anks for the ability to impede NF-κB nuclear accumulation validated Ank1 and Ank6 as having the most robust phenotypes. Next, RIPK3 levels during *O. tsutsugamushi* infection was examined. Contrary to that reported for cowpox infection, in *O. tsutsugamushi* infected cells, RIPK3 levels increase in a time-, bacterial dose-, and bacterial protein synthesis-dependent manner. These data suggest that *O. tsutsugamushi* infection potentially induces necroptosis and that it and cowpox virus use similar yet distinct mechanisms to inhibit NF-κB.

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Quantitative analysis of growth and morphology in the obligate intracellular pathogen *Rickettsia parkeri*

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Members of the Spotted Fever Group (SFG) of the *Rickettsia* genus are prominent tick-borne pathogens, yet our understanding of their fundamental growth aspects is limited due to their obligate intracellular lifestyle. We therefore sought to develop quantitative tools to investigate the basic biology of *Rickettsia parkeri*, an SFG member that causes mild disease and thus makes it a tractable model to study in the laboratory. Our first goal was to measure the morphology of live *R. parkeri* cells over the course of infection. To do this, we leveraged strains of *R. parkeri* constitutively producing cytoplasmic fluorescent protein (either GFP or the super bright fluorescent protein AausFP1). Because cytoplasmic fluorescence reflects the shape of the cell, it can be used to measure bacterial morphology within live eukaryotic host cells. We infected human lung epithelial A549 cells with GFP-producing *R. parkeri (Rp*GFP), imaged at various times post infection, and used MicrobeJ software to measure cell length and width. We also plotted GFP distribution as a function of cell length to identify cells actively undergoing cell division. These analyses allowed us to conclude that cell morphology does not change appreciably over the first 48 hours of infection; however, the fraction of the population actively undergoing cell division decreases at 24 hours post infection, suggesting infection-stage specific changes in cell cycle progression. Next, we sought to develop a high-throughput method to measure population growth kinetics. We again used *Rp*GFP or *Rp*AausFP1 strains and measured fluorescence over time as a reflection of bacterial cell mass using an imaging plate reader. By taking measurements every 3 hours of 3 biological replicates in 24-well plates, we calculated doubling times of 5 to 7 hours. With these methods in hand, we asked if inhibiting the actin homolog MreB, an important regulator of the cell elongation machinery in many rod-shaped bacteria, impacted *R. parkeri* morphology or growth. Indeed, we found that pharmacological inhibition of MreB caused an increase in both *R. parkeri* cell width and doubling time in a dose-dependent manner. These novel and highly quantitative tools will allow us to elucidate mechanisms of morphogenesis and growth regulation in *R. parkeri* and related species.

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Ehrlichia chaffeensis TRP120 activates BMP2 signaling in monocytes

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Ehrlichia chaffeensis is a gram-negative, obligately intracellular bacterium and the etiologic agent of human monocytic ehrlichiosis (HME)[1]. *E. chaffeensis* infects host monocytes and evades innate immune responses by utilizing secreted effector proteins that manipulate the host cell. The 120-kDA tandem repeat protein, TRP120, is a key effector protein that functions as a nucleomodulin, ubiquitin ligase, and ligand mimetic[2–4]. The intrinsically disordered regions within TRP120 contain short linear motifs (SLiMs) which activate evolutionarily conserved cellular pathways. Studies have demonstrated that TRP120 activates the Notch [5,6] and Wnt [7] signaling pathways to promote intracellular survival of *E. chaffeensis*. Preliminary data from our laboratory also demonstrates that *E. chaffeensis* activates the Sonic Hedgehog signaling pathway, likely through similar mechanisms. Our laboratory has discovered that TRP120 exhibits sequence homology with bone morphogenetic protein 2 (BMP2), a ligand of the BMP2 signaling pathway. BMP2 is an osteoinductive cytokine that serves a pathological role in vascular inflammation and cancer [8]. We hypothesize that *E. chaffeensis* TRP120 contains a BMP2 short linear motif (SLiM) mimetic that activates BMP2 signaling to stimulate chemokine secretion and induce monocyte chemotaxis thereby promoting infection. To determine the relationship between *E. chaffeensis* and the BMP2 signaling pathway, THP-1 cells were infected with crude *E. chaffeensis* and harvested between 0-48 hours post-infection. Immunofluorescence analysis demonstrated an upregulation of BMP2 pathway components, phosphorylated-SMAD1/5/8 and SMAD4, suggesting that *E. chaffeensis* activates BMP2 signaling. An NCBI Blast search revealed a SLiM within TRP120 that mimics BMP2. Gene expression studies were performed to determine if the BMP2 SLiM can modulate the expression of BMP2 target genes. RT-qPCR data showed an increase in ID-1 transcript levels after 30 min, similar to control cells treated with recombinant BMP2. Taken together, these findings suggest that *E. chaffeensis* may hijack the BMP2 signaling pathway to promote monocyte infection.

CteG mediated control of centrin-2: Getting to the center of how chlamydia manipulates the centrosome cycle

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Chlamydia trachomatis is the most prevalent bacterial sexually transmitted infection, as well as the leading cause of non-congenital blindness worldwide. Infections are often asymptomatic, leading to severe consequences such as pelvic inflammatory disease, ectopic pregnancy, and sterility. Moreover, individuals with current or prior infections are at an increased risk of developing ovarian or cervical cancers. *C. trachomatis* infection has been correlated with blocked cytokinesis, supernumerary centrosomes, and multipolar spindles; however, the mechanisms behind how *C. trachomatis* induces these cellular abnormalities are unknown. From the confines of its inclusion, *C. trachomatis* must engage numerous host organelles and signaling pathways to generate a niche that is permissive for intracellular replication. To accomplish these feats, *C. trachomatis* releases an array of proteins into the host cell via a type III secretion system (T3SS). Using affinity purification-mass spectrometry, we show that one such T3SS effector protein, CteG, binds to centrin-2 (CETN2), a key structural component of centrosomes and regulator of centriole duplication. Using sequential truncations, we have determined that the C-terminus of CteG binds to the C-terminus of CETN2, a region containing a key calcium binding domain. Significantly, deletion of CteG leads to normal centrosome number, indicating that *C. trachomatis* infection-induced centrosome amplification occurs in a CteG-dependent manner. Uniquely we have identified the first effector to target centrins and have begun to address how *C. trachomatis* induces gross cellular abnormalities during infection. Understanding the mechanistic underpinnings of the effect of CteG-CETN2 interaction, its impact on the centrosome cycle, and the long-term consequences this has on host cells could explain why chlamydial infection leads to an increased cancer risk.

The Attraction of *Dermacentor variabilis* to road edge habitat: evaluating the role of sensory organs in the distribution of American dog ticks in a field

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Ticks have a wide variety of sensory organs that facilitate host detection. Haller's organs allow ticks to sense infrared (IR) radiation and CO_2 emissions in the environment, emanating from hosts. Ocelli act as primitive eyes and allow ticks to sense visible light. These senses may attract ticks to road edge habitat, where IR radiation and CO_2 emissions tend to be high. We investigated the role of the Haller's organ and ocelli in the attraction of adult *Dermacentor variabilis* toward road edge habitat. Ticks were collected from Pennsylvania State Game Lands Number 169, separated into three groups (n=50 per group), and modified in the following manner: group 1 had the Haller's organs surgically removed; group 2 had ocelli painted with nail polish to make light detection impossible; and group 3 consisted of unmodified ticks (control). All tick groups were marked with a unique color of fluorescent paint and released 7.5 meters from the road edge at four study sites within SGL 169. Tick movements were tracked at night using ultraviolet lights, tick positions were recorded using flags, and measurements were taken to track tick movement in relation to the release point and the road edge (surface temperatures and ambient CO_2 levels were recorded at the road edge and in the field). Mixed-effects models were used to investigate the potential significance of tick proximity to the road edge between the groups. Results demonstrated the control and no ocelli groups were significantly closer to the road edge than no Haller's groups over time ($p = 0.023$) at sites 1 and 2. Our results indicate the Haller's organs of adult *D. variabilis* play a vital role in their attraction to road edge habitat. Laboratory simulations of the field experiment are currently underway to observe tick attraction to various IR radiation wavelengths.

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Role of *Sca4* in the dissemination and transmission of *Rickettsia parkeri* in *Amblyomma maculatum*

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Rickettsia parkeri, a member of spotted fever group, was historically believed to disseminate to host tissue by a cell-to-cell spread mechanism which involves actin-based motility to provide a physical force to spread. The current understanding is *R. parkeri* uses a different mechanism by secreting effector Sca4 to modulate protrusion engulfment by manipulating vinculin-dependent intercellular tension. However, the interactions of Sca4 in the tick infection model are unknown. Using *R. parkeri* lacking functional Sca4 (*R. parkeri sca4::tn*), we compared replication, growth kinetics and cell-to-cell spread *in vitro*, in a tick cell line. In order to assess the role of Sca4 in dissemination in the tick host and subsequent transmission, *Rickettsia*-free *Amblyomma maculatum* were exposed to wild type (WT) or *R. parkeri sca4::tn*. Individual tick saliva and tissues were analyzed by qPCR and IFA at 3 and 7 days post-exposure and a portion were allowed to feed to repletion. Guinea pig blood and skin were collected to access rickettsial transmission. Compared with WT bacteria, the *sca4::tn* mutant had slower growth kinetics *in vitro* and less bacterial load *in vivo.* In addition, the WT *sca4* expression in ISE6 cells and tick salivary glands suggests that Sca4 is involved in rickettsial infection of the arthropod host.

Identification and functional analysis of *Coxiella burnetii* dugway strain-specific type IV secretion system effector proteins

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Coxellia burnetii is a gram-negative intercellular bacterium that is the causative agent of Q fever. This disease presents as either an acute flu-like illness or persistent, focalized infection. Livestock are the primary natural reservoir of *C. burnetii* and majority of human infections are acquired by inhalation of contaminated aerosols derived from these animals. In 1957, at the Dugway Proving Grounds, Utah, USA, genetically unique strains of *C. burnetii* were isolated from wild deer mice and kangaroo rats. Despite retaining a primary virulence factor, full-length lipopolysaccharide, these Dugway strains were reported to exhibit avirulence *in vivo*. We confirmed this observation in a guinea pig intraperitoneal infection model, with Dugway-infected animals displaying similar trends in body temperature and weight as that of saline mock infected animals, diverging from the positive, virulent infection control. Additionally, a Dugway ∆*dot/icm* strain was generated that lacked the type IV secretion system (T4SS) and infection with this strain resulted in a similar lack of virulence. Dugway strains display the largest *C. burnetii* genomes which include T4SS effector genes. *C. burnetii* T4SS effector proteins are important for several bacterial and host functions, including host cell survival and immune modulation. We hypothesize that these unique T4SS effector proteins may contribute to Dugway's avirulence via interaction with host cells. After confirming Dugway avirulence *in vivo*, we identified 23 Dugway-specific putative T4SS effector genes and confirmed bacterial secretion by an adenylate cyclase reporter assay. In order to determine the potential role(s) of these effector proteins in Dugway avirulence, we designed fluorescently tagged Dugway effector fusion proteins to be utilized in mammalian ectopic expression assays. Select effector proteins have been analyzed for ectopic expression and generally display non-specific localization patterns. Additional RNA sequencing analysis is underway. Together, this work will lead to improved functional understanding of these proteins during infection and elucidation of important factors influencing host-pathogen interactions.

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Host transcriptomic profiling of CD-1 outbred mice with severe clinical outcomes following infection with *Orientia tsutsugamushi*

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Orientia tsutsugamushi is an obligately intracellular bacterium with endothelial tropism and can cause mild to lethal scrub typhus in humans. No vaccines are available for this emerging and severely neglected infection. While inbred mice are the focus of previous studies, such models have intrinsic limitations. Development of suitable mouse models that better mimic human diseases is in great need for immunologic investigation and future vaccine studies. This study is aimed at establishing scrub typhus in outbred CD-1 mice and defining immune biomarkers for disease severity. CD-1 mice received *O. tsutsugamushi* Karp strain via the i.v. route; major organs were harvested at 2-12 days post-infection for kinetic analyses, including lung immune gene profiling by using NanoString analysis. Surprisingly, we found that for our given infection doses, CD-1 mice were significantly more susceptible (90-100% death) than were inbred C57BL/6 mice (0-10% death), due to extensive bacterial dissemination and growth. Gross pathology of infected CD-1 organs revealed features that mimicked human scrub typhus, including pulmonary edema, signs of interstitial pneumonia, perivascular lymphocytic infiltrates, and vasculitis. Importantly, CD-1 mice displayed a Th1/cytotoxic, but Th2 suppressed, profile in the lungs, including a set of novel biomarkers not previously investigated in other scrub typhus models. Bio-plex analysis of serum cytokine and chemokine levels revealed that CD-1 mice developed a robust inflammatory response, which correlated with immune cell recruitment during the acute phase of the disease. This study provides an important framework indicating the value of CD-1 mice for scrub typhus research, especially for the investigation of host innate responses and immune dysregulation. This preclinical model is particularly useful for future translational and vaccine studies for severe scrub typhus.

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Roles of rickettsial outer membrane protein B (OmpB) in tick vector infections

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Rickettsia parkeri is an emerging pathogen of public health concern and is transmitted to humans by the Gulf Coast tick, *Amblyomma maculatum*. During infection of mammalian cells, rickettsial outer membrane protein B (OmpB) is associated with adhesion, invasion, and infection of mammalian host cells. More specifically, in macrophage cells, OmpB is required for *R. parkeri* to evade targeting by host cell autophagy, a component of the innate immune system. While the function of rickettsial OmpB is being elucidated in mammalian hosts, the role of this protein in arthropod infection is unknown. We hypothesized that if OmpB is essential in the vector, then disruption of *R. parkeri* OmpB will result in a defective infection or transmission phenotype. *R. parkeri* wild-type (strain Portsmouth), *R. parkeri ompB*^{STOP}::tn (non-functional OmpB) were infected in either tick-derived ISE6 for *in vitro* internalization and growth kinetics analysis. *In vivo*, tick infection and transmission bioassays were performed by capillary feeding rickettsiae into the naïve *A. maculatum* ticks and fed on rat models. Internalization assays showed a temporary reduction of invasion function in *R. parkeri ompB*^{STOP}::tn infected tick cells and returned to similar ratio of invasion as wild-type after 90 minutes post-infection. Growth kinetics were indistinguishable between the *R. parkeri* wild-type and *ompB*^{STOP}::tn strains in cultured ISE6 cells. However, ticks exposed to wild-type *R. parkeri* had greater rickettsial loads in all organs (midgut, salivary glands, ovary) compared to the ticks exposed to the *ompB*^{STOP}::tn. This study reveals that while *R. parkeri* OmpB is dispensable for bacterial growth in cultured tick cells, it enhances infection in the tick host *in vivo*. Understanding the role of OmpB in tick vector may lead to strategies for targeting this protein to control this tick-borne disease.

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Tissue-specific transcriptional responses to *Rickettsia rickettsii* infection in mice

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An understanding of the interactions between host cells and pathogens is crucial for identifying molecular mechanisms that allow the pathogen to survive and cause disease in a host. *Rickettsia rickettsii*, the etiological agent of Rocky Mountain spotted fever, is an intracellular bacterium that replicates within eukaryotic cells, presenting novel "inside-out" challenges for host response mechanisms. During *R. rickettsii* infection, the bacterium adheres to the host cell surface and induces its own engulfment. Although various proteins involved in invasion have been identified, these interactions are not yet fully understood – particularly as they relate to colonization of different host tissue and specific cell types. To address this gap in knowledge, we have developed a mouse model to analyze host-pathogen transcriptional profiles from different host tissues during *in vivo* infection with *R. rickettsii*. In our preliminary study, we reported a surprisingly large number of differentially regulated host transcripts in spleen tissue compared to liver tissue, with only a handful of transcripts in common. Genes of interest were then assessed for their predicted subcellular localization and pathway involvement. Promising targets including rickettsial *Sca* genes were examined further in cultured endothelial cells and differentiated macrophages to identify changes in expression specific to host cell type, with particular focus on genes that encode surface proteins and receptors. These target genes will be compared to genes differentially expressed in closely related, non-disease-causing *Rickettsia montanensis*. Genes that are not expressed in *R. montanensis* but are expressed in *R. rickettsii* during infection will be leveraged for RNAi knockdown studies to determine the specific host-pathogen interactions between rickettsial pathogens and host cells. This work will provide valuable insights into the interplay between pathogen and host cells during the establishment of infection and guide efforts to develop novel vaccines and diagnostics to combat rickettsial diseases.

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Novel detection of *Rickettsia* species in *Ambylomma maculatum* found in Callaway County, Missouri

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Amblyomma maculatum, commonly known as the Gulf Coast tick, is an ornate tick typically found along the Atlantic and Gulf coast regions. However, the range of this species is increasingly expanding northward. *Amblyomma maculatum* is a known vector of *Rickettsia spp.,* of which several species are pathogenic to humans and can cause symptoms such as fever, rash, and muscle aches. With increased efforts to restore prairie habitat in the Central Plains Region, there may increase potential habitat for ticks via mechanisms of providing suitable vegetation and habitat for hosts ticks feed upon and are potentially highly competent reservoirs for tick-borne pathogens. We sought to understand the role of prairie reconstruction in the presence of ticks and of tick-borne pathogens. From 2020-to 2021, we collected American Dog and Lone Star ticks from a reconstructed and remnant prairie in Callaway County, Missouri; to our surprise, we discovered several Gulf Coast ticks here as well. Upon testing the ticks for the Rickettsia 16S rRNA gene through PCR, we found over 50% of ticks tested positive, all of which were located in the reconstructed prairie. Our collected data shows the first detection of *Rickettsia spp.* in *A. maculatum* in Central Missouri, primarily in reconstructed prairies. With expanded interest in the function of prairie habitats concerning disease reservoirs and transmission pathways of pathogens via vectors, there is a greater need for proactive surveillance of tick-borne diseases.

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Cryptic genes for interbacterial antagonism distinguish *Rickettsia* species infecting blacklegged tick from other *Rickettsia* pathogens

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Genus *Rickettsia* (*Alphaproteobacteria*: Rickettsiales) encompasses a plethora of obligate intracellular species with predominantly ciliate and arthropod hosts. The best-known *Rickettsia* species are pathogens transmitted to mammals by blood-feeding arthropods. Mammalian pathogenicity seems to have evolved from basal, non-pathogenic host-associations; however, some non-pathogenic species are also found closely related to pathogens. One such species, *Rickettsia buchneri*, is found in high prevalence in the blacklegged tick, *Ixodes scapularis*. While *I. scapularis* transmits several microbial pathogens to humans, it does not transmit *Rickettsia* pathogens, consistent with *R. buchneri* being the predominantly reported *Rickettsia* infection of blacklegged tick. We hypothesize that *R. buchneri* has established a mutualism with *I. scapularis*, possibly blocking tick superinfection with *Rickettsia* pathogens. Studies evaluating *R. buchneri*-*I. scapularis* mutualism would benefit from development of a *R. buchneri*-specific assay to adequately assess infection frequency in blacklegged tick populations. Accordingly, we performed comparative genomics analysis to identify an *R. buchneri* gene (*REIS_1424*) not present in other *Rickettsia* species present throughout the geographic range of *I. scapularis*. Subsequent bioinformatic and phylogenomics approaches were employed to propose a function for the hypothetical protein (263 aa) encoded by *REIS_1424*. REIS_1424 was determined to have few analogs in other Rickettsiales genomes and greatest similarity to proteins from distantly related non-proteobacterial genomes. While this cohort of proteins varied greatly in size and domain composition, possessing characteristics of Recombination hotspot (Rhs) and contact dependent growth inhibition (CDI) toxins, similarity was limited to a proximal C-terminal domain (~145 aa). This domain was named CDI-like/Rhs-like c-terminal toxin (CRCT). Such proteins are often found as toxin-antidote (TA) modules, leading us to interrogate REIS_1423 (151 aa) as a putative antidote encoded upstream of *REIS_1424*. REIS_1423 is highly conserved in length and composition to proteins encoded upstream of CRCT domain-containing proteins; accordingly, these proteins were named CDI-like/Rhs-like C-terminal toxin antidotes (CRCA). *R. buchneri*

expressed both *REIS_1423* and *REIS_1424* in tick cell culture, and PCR was employed to show *REIS_1424* is a unique gene that will be useful for probing *I. scapularis* populations for specific *Rickettsia* infections. Finally, an exhaustive search of *Rickettsia* genomes uncovered several divergent CRCT/CRCA modules in various states of conservation, but with only *R. buchneri* and related endosymbionts in the Tamurae/Ixodes Group rickettsiae having TA modules predicted to be functional. We hypothesize that *Rickettsia* CRCT/CRCA modules circulate in the intracellular mobile gene pool, possibly arming rickettsiae for battling other microbes for arthropod host colonization. While the functional significance of these modules remains to be tested, the module unique to *R. buchneri* serves as a marker to positively identify *R. buchneri* infection and begin deciphering the role this endosymbiont plays in the biology of the blacklegged tick.

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Anaplasma marginale infection of *Dermacentor andersoni* primary midgut cell culture is dependent on fucosylated glycans

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Tick midgut is the primary infection site required by tick-borne pathogens to initiate their development for transmission. Despite the biological significance of this organ, cell cultures from midgut tissues are unavailable. To study the mechanism of *Anaplasma marginale*-tick cell interactions, we successfully developed an *in vitro Dermacentor andersoni* primary midgut cell culture system. Midgut cells were maintained for up to 120 days. We demonstrated the infection of *in vitro* midgut cells by using an *A. marginale* omp10::himar1 mutant with continued replication for up to 10 days post- infection. *Anaplasma marginale* infection of midgut cells regulated the differential expression of tick α-(1,3)-fucosyltransferases A1 and A2. Silencing of α-(1,3)- fucosyltransferase A2 in uninfected midgut cells reduced the display of fucosylated glycans and significantly lowered the susceptibility of midgut cells for *A. marginale* infection, suggesting that the pathogen utilizes core α-(1,3)-fucose of N-glycans to infect tick midgut cells. This is the first report using *in vitro* primary *D. andersoni* midgut cells to study *A. marginale*-tick cell interactions at the molecular level. The primary midgut cell culture system will further facilitate the investigation of tick-pathogen interactions, leading to the development of novel intervention strategies for tick-borne diseases.

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Development of a CRISPR interference system for genetic manipulation of the bacterial human pathogen *Coxiella burnetii*

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Coxiella burnetii is an obligate intracellular human pathogen and zoonotic agent of Q fever. As a fastidious, slow-growing bacterium, genetic manipulation of *C. burnetii* often proves difficult. The most common methods for site-directed mutagenesis in *C. burnetii* include the cre-lox recombination and “loop-in loop-out” methods. While effective, these methods are often time-consuming and laborious with no guarantee of successful transformants. As such, we have developed a directed transcriptional repression knock-down approach to genetic manipulation in *C. burnetii* that functions via a mini-Tn7-based CRISPR interference system under the control of a chemical inducer. In combination with amino acid auxotrophic selection in the chemically defined medium ACCM-D, this method of manipulation has proven quick (< 1 month to transformants) and effective. As a proof-of-concept, we have developed a *C. burnetii* strain harboring a CRISPR interference-mediated knock-down of the *icmD* gene, coding for an essential component of the type IV-B secretion system apparatus. When chemically induced, this strain has growth and visual phenotypes in tissue culture infections similar to those of an isogenic *icmD* mutant strain. Additionally, we have developed a method for successful complementation of knock-down phenotypes, as well as a strategy for simultaneous transcriptional repression of multiple genes from a single transformation, as demonstrated by an *icmD* – *scvA* double knock-down strain. We hope that this inducible method for genetic manipulation will allow for more precise examination of *C. burnetii* virulence and morphogenesis.

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Analysis of a new type IV secretion system effector of Anaplasma phagocytophilum

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Human granulocytic anaplasmosis (HGA) is an emerging tick-borne infectious disease that causes a potentially fatal, severe influenza-like illness. The causative agent of HGA, *Anaplasma phagocytophilum* (*Aph*), is a Gram-negative obligatory intracellular bacterium that infects mammalian and tick cells. Details of how *Aph* infects, survives, and proliferates in these cells remain mostly unknown. The Type IV secretion system (T4SS) is utilized by *Aph* to directly inoculate bacterial molecules into human cells to manipulate host cells for its survival and proliferation. Based on bioinformatics analysis and bacterial two-hybrid system by using *Aph* VirD4 as bait, a hypothetical protein APH0874 was identified as a potential T4SS effector. To experimentally validate APH0874 as a bona fide T4SS effector, *Aph* mutant expressing FLAG-tagged C-terminal APH0874 (FLAG-APH0874C) containing T4SS secretion signal was constructed by Himar1 transposon mutagenesis system. DeltaVision microscopy showed that FLAG-APH0874C is produced by the *Aph* mutant, secreted into the host cytoplasm, and localized to the *Aph* inclusion membrane. By immunofluorescence labeling using rabbit IgG against recombinant C-terminal APH0874, native APH0874 was demonstrated to be indeed secreted and localized to *Aph* inclusions. By transfecting uninfected or *Aph*-infected RF/6A cells with GFP-tagged full length- or C-terminal half of APH0874, DeltaVision microscopy showed that both full-length and C-terminal fragment of APH0874 localize to the Golgi apparatus with cis-Golgi marker GM130 in uninfected cells, and on *Aph* inclusion membranes in infected cells. These results demonstrated APH0874 is a newly verified *Aph* T4SS effector and may play an important role in *Aph* infection by localizing on *Aph* inclusions. Further studies on analyzing molecular mechanisms by which APH0874 localizes on *Aph* inclusions and Golgi apparatus will shed light on our understanding of roles of this newly identified T4SS effector APH0874 in *Aph* proliferation and its inclusion biogenesis.

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Symbiont control of arthropod vector autophagy: Insights from the blacklegged tick, *Ixodes scapularis*

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Ticks, as obligate blood-sucking arthropods, can cause substantial public health and economic burdens by direct feeding behaviors and transmitting a broad range of viral, bacterial, and protozoan pathogens to hosts. To date, approximately 90 known tick species are recognized as vectors responsible for spreading emerging infectious diseases throughout the world. For example, although approximately 95% of vector-borne disease cases reported in the USA are associated with ticks, the impact of all tick-borne illnesses remains mostly unquantified. In this study, we focused on the black-legged tick, *Ixodes scapularis*, a vector of seven human pathogens, including those causing Lyme disease, anaplasmosis, babesiosis, *etc*, and utilized *Rickettsia buchneri* (*Rb*) (Rickettsiales: Rickettsiaceae), a spotted fever group rickettsia, and non-pathogenic symbiont in *I. scapularis* to investigate the role of autophagy in symbiont and tick interactions. Using RNA FISH in situ hybridization, we found that *Rb* is broadly distributed in the female tick ovary and possibly participates in oocyte development. *Rb* infection also regulated autophagy-related genes expression and led to an increase in autophagosome accumulation *in vitro* (tick cells). Transmission electron microscopy (TEM) revealed *Rb* enveloped in autophagic vesicles both *in vitro* and *in vivo* (female tick ovary). We also observed the intracellular colocalization of lysosomes/ autolysosomes and *Rb*, suggesting *Rb* infection could prompt formation of tick autophagosomes. Furthermore, chemicals
(autophagy inhibitors and inducers), antibiotics, and RNAi will be utilized to determine whether *Rb* induced-autophagy participates in tick reproduction. This research on how *Rb* affects its *I. scapularis* tick vector will provide more clues to solve the tick-symbiont interaction "Jigsaw puzzle."

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Comparative transcriptomics identifies *Coxiella burnetii* survival strategies in continuous axenic media growth

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Coxiella burnetii (*Cb*) is an obligate intracellular pathogen in nature and causes acute Q fever and chronic disease. The bacterium typically infects macrophages, and successfully propagates in a parasitophorous vacuole (PV) that has the properties of a phagolysosome. To identify genes and proteins crucial to their normal intracellular growth lifestyle, we applied a "Reverse Evolution" approach where the avirulent NMII strain of *Cb* was grown for 67 passages (67 weeks) in chemically defined Acidified Citrate Cysteine Media- D (ACCM-D). Gene expression patterns from various passages were compared to passage 1, which followed intracellular propagation of the bacteria. Transition of *Cb* into axenic media over the first 10 passages revealed a decrease in *Cb* infectivity and growth within HeLa cells. Comparative transcriptomic analysis of passages (out to passage 67) of *Cb* identified a marked downregulation of structural components of the type 4B secretion system (T4BSS), and the general secretory (sec) pathway. Additionally, 14 out of 118 previously identified genes encoding T4BSS effector proteins were significantly downregulated. These are predicted to be involved in signal transductions, carbohydrate metabolisms, posttranslational modification, and lipid metabolisms. Additional downregulated pathogenicity determinant genes included several chaperones, LPS, and peptidoglycan biosynthesis genes. A marked downregulation of central metabolic pathways including glycolysis, TCA cycle, electron transport chain and FA biosynthesis was observed, which was balanced by an upregulation of genes encoding transporters. This pattern reflects the richness of the axenic media and diminishing anabolic, and ATP-generation needs in the media. Further, we identified 30 hypothetical proteins (13 cytoplasmic, 8 inner membrane, 1 extracellular, 1 periplasmic and 7 with unknown localization) that were significantly downregulated. These may represent determinants with roles in intracellular survival. Our work indicates that *Cb* gene expression changes significantly following acclimation to axenic media and provides us with genes vital in *Cb* intracellular growth for further investigation.

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Ca2+ regulation during host-*Rickettsia* interaction

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Human Rickettsioses are potentially life-threatening infections caused by the gram-negative pathogens of the genus *Rickettsia*. Treatment for *Rickettsia* infection is limited to doxycycline with notable failure rates if diagnosis and treatments are delayed. With genomic evidence of past acquired antibiotic resistance and treatment failures, alternative approaches for the treatment of Rickettsiosis are required. One such approach is host-directed therapeutics (HDT) through repurposing FDA approved drugs. As an obligate intracellular organism, *Rickettsia* relies entirely on their host for survival, thus making host directed therapeutics a valid approach. Multiple Calcium Channel Blockers (CCBs) have been identified from previous drug screens to exhibit inhibitory effects on the growth of *Rickettsia* in tissue culture models. My goals are to examine how CCBs protect the host against *Rickettsia* infections and why host Ca^{2+} signaling is essential for *Rickettsia* proliferation. Previously performed preliminary screening of a library of FDA-approved non-antibiotic drugs has revealed a group of Calcium Channel Blockers (CCBs) that exhibit inhibitory effects on *Rickettsia* growth in tissue culture. When used alternatively, they are proposed to modulate the mammalian calcium signaling system to exert antibacterial effects, as has been established in *Listeria monocytogenes* and *Mycobacterium tuberculosis*. However, the exact role of Ca^{2+} during *Rickettsia* infection has yet to be characterized. We employed chemical and biological approaches to determine the mechanisms of CCB efficacy and the role of Ca^{2+} during *Rickettsia* infection of endothelial cells. The efficacy of CCBs during endothelial *Rickettsia* infection is established and the potential cellular stages of host-*Rickettsia* infection that CCBs could affect are deduced. Cytoplasmic Ca^{2+} influx is observed upon induced infection with pathogenic *Rickettsia* species and intracellular calcium accumulates throughout infection. Our investigation defines the effects of CCBs on *Rickettsia* endothelial cell infection and demonstrates that Ca^{2+} is a regulatory component during *Rickettsia*-host infection.

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Lessons from an obligate stealth pathogen: Multiple ways to "skin a cat"

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The ability of many obligately intracellular pathogens to survive in monocyte/macrophage is an essential virulence strategy. *Coxiella burnetii*, the etiologic agent of acute Q fever and occasional chronic manifestations, is becoming well-characterized as one of the most prolific in ways to manipulate the host to create a permissive replication compartment. Part of this strategy is by dampening innate response mechanisms to prevent macrophage activation which would canonically lead to expression of toxic reactive oxygen and nitrogen intermediates and create a non-permissive host. Recent studies have identified several type 4 secretion-dependent effectors which may modify various pathways of innate response. Identification of putative effectors that dampen a key central response pathway (NFkB regulon) are presented in detail of recently published or recent results for at least 6 unique effectors. The identification of each was initially made using either ectopic expression in reporter macrophage model lines or as a macrophage specific replication requirement. Verification and molecular analysis varied between effectors and mechanistic models depended on multiple readouts including cellular localization and binding partner identification. Several effectors appear to modulate NFkB regulon by interaction with upstream signaling cascade primarily in the cytoplasm while others enter the nucleus and alter transcriptional events. The contribution of each effector to the pathogenic strategy for Q fever will ultimately depend on testing genetic deletion or active site mutations of each determinant in virulence animal models.

Downregulated expression of MHC-I in *Anaplasma phagocytophilum*-stimulated human monocyte-derived macrophages: A role for *NLRC5* isoforms?

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Human anaplasmosis is a tick-borne disease caused by the obligate intracellular rickettsia *Anaplasma phagocytophilum* (*Aph*). Severe infections in humans are characterized by macrophage activation syndrome (MAS) which is related to cytotoxic lymphocyte (CTL) dysfunction or lymphopenia. *Ex vivo* mouse studies showed that *Aph*-stimulated splenic antigen-presenting cells are defective in activating NK, NKT, and CD8 T cell cytotoxicity, suggesting lack of APC MHC class I presentation. MHC-I expression is governed the transcriptional regulator NLRC5. RNA-seq profiling of *Aph*-infected ATRA- differentiated HL-60 cells reveals alternative splicing of *NLRC5* that doubles nonsense-mediated decay isoform transcripts (*NLRC5*-010), which are not translated and would diminish MHC-I expression. We hypothesize that defective CTL activation results from *NLRC5* alternative splicing in *Aph*-stimulated APCs leading to reduced MHC-I expression. We stimulated human monocyte-derived macrophages (hMDM) with live and heat-killed cell-free *Aph* and examined MHC-I expression by flow cytometry, immunofluorescence microscopy, and RNAseq. *Aph* in hDMDs were non-viable, but a dose-dependent reduction in MHC-I expression with live but not heat-killed *Aph* (MOI 100:1 [$p<0.01$] and MOI 1000:1 [$p<0.001$]) vs. mock-stimulated control was observed. Fluorescence microscopy confirmed these findings. RNAseq targeting *NLRC5* demonstrated an increase in transcription in live *Aph*-stimulated hMDMs at MOI 1:100 (32.8 TPM ± 2.6 SEM, $p=0.046$) compared to controls (24.3 TPM±0.9). The increase was in part the result of isoform alternative splicing and expression of the nonsense mediated decay isoform *NLRC5*-010, as previously described with HL-60 cell infection. These results show that MHC-I expression is reduced in APCs stimulated by live *Aph*, in the absence of *Aph* growth, and that reduced expression could result from *NLRC5* alternative splice isoforms that are unable to be translated or have regulatory functions. Whether reduced MHC-I expression results directly from changes in *NLRC5* isoforms with live *Aph* stimulation and whether MHC-I downregulation results in diminished CTL activation need further investigation.

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An in vitro antibiotic treatment algorithm to eliminate mycoplasma contamination from rickettsial cultures

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Mycoplasma contamination is a universal problem in bacterial cell culture. These prolific organisms thrive in the extracellular environment in vitro and can persist in cell lines indefinitely without treatment. In mycoplasma-contaminated rickettsial cultures, laboratory animals have historically been used to clear the contamination. However, this method requires the sacrifice of live animals and is expensive, labor intensive, and not always effective. Antibiotics in cell culture represent an alternate method to eliminate contaminating *Mycoplasma* species. Daptomycin, a relatively new cyclic lipopeptide antibiotic, has shown promise against mycoplasmas in contaminated rickettsial cultures but can be prohibitively expensive for many applications, costing several thousand dollars to clear a single isolate at the currently recommended antibiotic concentration of 256 mg/L. Other less expensive antibiotics have also been used to clear some *Mycoplasma* species from rickettsial cultures, including lincomycin, clindamycin, and gentamicin. Nonetheless, antibiotic susceptibilities can vary considerably among species and strains of *Mycoplasma*, such that one regimen may be insufficient for clearing multiple infected isolates. We present here the results of the methodical testing of these antibiotics against various *Mycoplasma* species contaminating cultures from the rickettsial isolate collection at CDC and suggest a rational algorithm for in vitro antibiotic treatment of mycoplasmas infecting rickettsial cultures.

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Rickettsioses identified as a cause of febrile illness requiring hospitalization at two hospitals in Uganda

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Background

Rickettsial illness is suspected to be a common cause of febrile illness in sub-Saharan Africa, but the burden of severe illness is unclear. We describe rickettsial IFA and PCR results from an ongoing acute febrile illness protocol of hospitalized adults from two sites Uganda.

Methods

From August 7th, 2019, to August 11th, 2020, febrile participants admitted to two regional referral hospitals western and central Uganda were enrolled. Serum IFA was performed to measure spotted fever group (SFG) rickettsia, typhus group (TG) rickettsia, and *Orientia* IgG titers with baseline (acute) and one-month (convalescent) samples. Seroprevalent subjects were indicated if there was a ≥ 1:128 titer in either serum sample. A four-fold rise in titer between acute and convalescent samples was considered a seroconversion. RT-PCR was performed using the baseline whole blood sample targeting broad 16S ribosomal RNA (broad-range TG and SFG sequence), and positive results were followed by quantitative PCR targeting 17-kDa DNA PCR TG gene sequence), 56-kDA (*Orientia* gene), and *ompA* (SFG gene).

Results

Acute and convalescent samples were available from 67 participants (64.2% female; median 35.6 years of age) who presented after a median 4.7 days of symptoms. Seroprevalence was high at 46.3% for SFG, 10.5% for TG, and 27.3% for *Orientia*. Seroconversions were observed due to SFG rickettsia (N=6; 9.0%) and TG rickettsia (N=2; 3.0%), but none for *Orientia*. There were 2 participants with positive 16S rRNA RT-PCR testing: one TG PCR positive and TG IFA seroconverted and the other SFG

PCR negative but SFG IFA seroconverted. Both were negative for Orientia with PCR.

Conclusion

In this cohort in Uganda, SFG and TG rickettsia led to febrile illness requiring hospitalization. Rickettsia seroprevalence was high including Orientia IgG. Further research to define the burden of rickettsial illness and Orientia circulation are needed in this region.

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Investigating interferon signaling as a key determinant of human spotted fever disease

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Arthropod-borne pathogens often use animal reservoirs to transmit themselves horizontally in the wild. In many cases, rodent reservoirs have high resistance to infection, yet human ‘dead end’ hosts can develop serious and sometimes lethal disease. The molecular explanation for the discrepancy in human versus rodent susceptibility remains unclear. Our lab studies this phenomenon with the obligate cytosolic tick-borne pathogen *Rickettsia parkeri*. *R. parkeri* causes mild human spotted fever disease characterized by a skin lesion (eschar) at the site of tick bite, yet wild type (WT) laboratory mice are highly resistant to infection. We hypothesize that the differences in susceptibility are due to highly protective innate immune responses in mice. Indeed, we find that in primary mouse macrophages in vitro, *R. parkeri* is potently restricted by either type I interferons (IFN-I) or interferon-gamma (IFN-g). IFN-I upregulates guanylate binding proteins and inducible nitric oxide synthase, two innate immune factors that protect the cytosol, which we found to target and restrict *R. parkeri*. Moreover, we found that mice lacking either IFN-I or IFN-g signaling resist infection by *R. parkeri,* whereas mice lacking both signaling receptors (*Ifnar*-/-*Ifngr*-/-) develop serious disease upon intravenous or intradermal infection. Intradermal delivery results in eschar formation that mimics human disease with kinetics similar to human systemic disease. These results suggest that the explanation for why humans develop disease whereas rodents are resistant to infection is due to differences in interferon signaling. Future studies seek to discover whether murine interferon-stimulated genes are more potent than human interferon-stimulated genes to uncover the molecular basis of human arthropod-borne disease.

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Anaplasma phagocytophilum HGE14 effectors: A viral tail

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The obligate intracellular bacterium *Anaplasma phagocytophilum* (*Ap*) elicits wide ranging effects on host cell biology such as diminished respiratory burst, inhibition of apoptosis, and altered chemokine and cytokine expression, to enable its survival. Effector proteins, deployed via its type IV secretion system (T4SS), are thought to have a central role in *Ap*-mediated host cell remodelling. Five Ap T4SS effector proteins have been identified: AnkA, Ats-1, APH0215, APH0177 and HGE14. HGE14 is a member of a family of proteins that have been used as serological markers of infection. No function has been determined but their nuclear localisation (of both heterologously expressed and native HGE14s), and the ability to diminish the respiratory burst of HL-60 cells in heterologous expression systems, suggest that HGE14s likely interact with host nucleoproteins and/or DNA to modulate host cell biology. We sought to define the function(s) of the HGE14 family. Protein sequence analysis indicates that, whilst all HGE14s contain an ordered N-terminal cysteine-rich core domain, some members also encode a C-terminal "tail" domain of variable length. These tail domains contain repeating motifs with similarity to those found in host proteins targeted by the nuclear deubiquitinase, USP7, suggesting that this host protein could be the target of tailed-HGE14s. We demonstrated specific interactions between HGE14 tail domains and the peptide-recognition domain of USP7 and present the structural basis for these interactions. These findings highlight that HGE14 tails function via molecular mimicry to target USP7, a protein which regulates a wide range of different processes and has key roles in cell cycle regulation and oxidative signalling. Remarkably, whilst USP7 is targeted by some DNA viruses (notably to inhibit apoptosis), to our knowledge, this is the first example of a bacterial effector targeting USP7 directly. This indicates that *Ap* employs strategies similar to some DNA viruses, but through a distinct mechanism.

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Anaplasma phagocytophilum transmission activates interferon signaling pathways in the skin

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Anaplasma phagocytophilum is the causative agent of Human Granulocytic Anaplasmosis (HGA), formerly known as Human Granulocytic Ehrlichiosis (HGE), which was discovered infecting a cluster of men in the upper Midwest. Recently, 5,655 cases were reported to the CDC in 2019, making HGA the second most common tick-borne disease in the US. In most cases, the illness is self-limiting, and patients will recover with and without antibiotic treatment. Symptoms will typically develop 1-2 weeks following a bite by an infected tick and can lead to hospitalization in around 36% of confirmed cases and mortality rates around 0.2%-1%. High levels of proinflammatory cytokines, such as Interferon (Ifn)-γ, Interleukin (IL)-12p70, and IL-10, are detected in HGA patients. *In vivo* experiments using murine models indicate that secretion of Inf-γ correlates with Stat1 phosphorylation. Stat1 knock-out in mice leads to increase bacterial loads and is associated with severe disease pathology. Nevertheless, the effect of *A. phagocytophilum* transmission on skin immune responses have not been explored. We investigated the differences in gene expression during transmission of *A. phagocytophilum* by ticks when compared to uninfected ticks. We observed the upregulation of several genes involved in interferon signaling. Interestingly, genes related to wound healing responses were downregulated during *A. phagocytophilum* transmission. Further, according to preliminary proteomic analysis of tick extracellular vesicles from *A. phagocytophilum* infected and uninfected ticks, 101 proteins showed significant difference at FDR<=0.05, suggesting that *A. phagocytophilum* infection changes tick extracellular vesicle cargo. Herein, we describe the effect that *A. phagocytophilum* transmission has on skin immune responses and in the salivary secretions of ticks. Given that the skin is the first site of infection for tick-borne pathogens, it is of interest to address how the localized skin immune response may affect bacterial establisment.

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Genomes of *Rickettsia rickettsii* strains representative of areas with elevated Rocky Mountain spotted fever risk

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Over the last several decades, the incidence of Rocky Mountain spotted fever has fallen in Western Montana where the disease was originally described. However, cases have steadily risen in Brazil and the Southwestern United States to Northern Mexico. In these regions, tick species other than *Dermacentor andersoni* are endemic and recognized as primary vectors of *R. rickettsii*, the etiological agent of Rocky Mountain spotted fever. To investigate whether genomic differences might exist due to regional variation or the specific sympatric vector, we sequenced the genomes of *R. rickettsii* Sheila Smith, AZ-5, Sao Paulo, and Taiaçu, integrating longer PacBio reads with shorter Illumina reads. The AZ-5 strain, recently isolated from a *Rhipicephalus sanguineous* tick, was included to reflect the increased number of spotted fever cases in Northern Mexico and the Southwestern United States. The Taiaçu and older Sao Paulo strains were isolated from *Amblyomma aureolatum* and *Amblyomma sculptum* respectively. The genomes were then contrasted to published *R. rickettsii* genomes.

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Potential vaccine targets in VirB10 of the *Anaplasmataceae*

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Tick-borne pathogens in the family *Anaplasmataceae* cause globally significant infections of domestic animals and humans, including the emerging U.S. diseases of anaplasmosis and ehrlichiosis and heartwater disease of livestock in Africa. Effective vaccines against the *Anaplasmataceae* are not available. Molecular investigations have revealed that immunodominant surface proteins of these organisms are often highly variable, which has stimulated the search for suitable conserved vaccine targets. One such target is the Type IV secretion system, a nanomachine spanning the two membranes of Gram-negative bacteria, responsible for translocation of effectors that enable colonization and survival of bacteria in the host cell. In *Escherichia coli* the crystallographic structure of the complete outer membrane complex of Type IV was determined and established VirB10 as comprising the outer membrane channel. Importantly, a two-helix C-terminal bundle of VirB10 was shown to be exposed extracellularly, demonstrating that it projects across the outer membrane of *E. coli*. In a recent investigation we observed that soluble recombinant VirB10 of *Anaplasma phagocytophilum*, used as a vaccine, was partially protective against challenge infection in a mouse model. Although little experimental structural information is available for VirB10 of the *Anaplasmataceae* the recent development of the AlphaFold structural prediction algorithms provides a route to compare VirB10 structures. Herein, we use AlphaFold to identify potential exposed vaccine targets within VirB10 of five different pathogens within the *Anaplasmataceae*.

Combined diagnosis of IFA and PCR increase the confirmation of suspected spotted fever cases

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Brazilian Spotted Fever (BSF) is an acute disease with high mortality, caused by *Rickettsia rickettsii* transmitted by infected ticks[1]. IFA is the most used serological method and also the most available in laboratory routine. Generally, IFA is recommended for cases with samples collected after 7 days of the onset of symptoms. The test should be repeated two weeks after the first one, to check if there is an increase in the antibody titer, confirming the evolution of the disease[2]. PCR testing is more effective in the first 10 days. The earlier the sample collection, the greater the chances of directly detecting the bacteria[3]. The date of onset of symptoms and the outcome of BSF are determining factors for choosing between IFA or PCR. The Central Laboratory of Public Health of FUNED (Ministry of Health of Brazil - MHB) performs diagnosis using IFA - IgM and IgG (*R. rickettsii* strain Taiaçu)[4] and PCR (*Omp*A and *Omp*B for this Taiaçu strain) for suspected cases, with samples collected with less than 12 days after symptoms. A survey was made of the results of these tests performed throughout 2019.From the 2,952 samples, 106 cases had the diagnosis confirmed by the IFA, PCR and both methodologies. In the evaluation of positive cases, it was observed that 57 samples were confirmed by IFA, 46 were considered positive by PCR and three had positive results by both. Comparing 2018 and 2019 (when both methods were used) the increase of confirmed cases was of 31.8%. Although PCR became an important tool for confirming suspected cases, serology still allows the diagnostic conclusion of a greater number of cases, due to its greater availability in the laboratory routine. Our conclusion is that the combination of these methods increase the sensitivity of the diagnosis.

The insect *Galleria mellonella* as an infection model to investigate pathogenesis of *Rickettsia*

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Animal models are the gold standards for studying virulence of *Rickettsia*; however, mammalian models of infection are expensive and require access to an ABSL facility. Since insects possess a complex innate immunity and advanced antimicrobial defenses, their responses to microorganisms may provide insight about initial infections of mammals. **The purpose** of this study was to evaluate susceptibility of *Galleria mellonella* larvae (GML) *to Rickettsia*.

Methods: Commercial GML were acclimated before infection with live *R. rickettsii* Sheila Smith. Controls included GML inoculated with boiled *Rickettsia* and K36 injection buffer, and non- injected larvae. Infected and control larvae were kept at 34°C and examined daily for changes in color, movement, and viability. Hemolymph and fat body were collected at each time point and used for microscopy and DNA and RNA extraction. SYBR Green PCR assays were used to quantify *Rickettsia* DNA. cDNA was synthesized from fat body RNA and tested for transcripts of GML antimicrobial peptide (AMP) genes using gene-specific qPCR assays.

Results: Hemocoel infection of GML with 10^6 plaque-forming units (PFU) of live *R. rickettsii* killed up to 30% of larvae between 48 and 72 hours of infection. This effect was mitigated by pretreatment of *Rickettsia* with therapeutic doses of antibiotics before infection. Surviving infected GML exhibited dose-dependent changes in vital signs. GML injected with heat-killed *Rickettsia* had similar responses including some death within 48 hours. Rickettsiae were microscopically observed in hemolymph of infected GML, and abundant transcripts of *Rickettsia* 16S rRNA and OmpA genes were detected in fat bodies at each time point tested. Transcription of six GML AMP genes varied exhibiting either two-peak profile or continued upregulation between 2 and 48 hours.

Conclusions: We demonstrated susceptibility of GML to *R. rickettsii*. Further studies will dissect individual pathways and investigate their relevance to innate immune responses in ticks and mammals.

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Utility of next-generation sequencing for etiological diagnosis of rickettsial diseases

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Acute stage diagnosis of rickettsial diseases is frequently unsuccessful due to non-specific flu-like clinical manifestations, insignificant hematologic changes and indeterminate serological findings. Traditional PCR-based methods provide reliable option for agent DNA detection if the proper assay is performed but can also suffer from relatively low sensitivity due to low rickettsiemia both early and late during infection. We report use of sensitive single molecule Next-Generation Sequencing (NGS) for diagnosis of spotted fever group rickettsiosis, scrub typhus (ST) and Q fever (QF) in three unrelated febrile patients presenting with fever of unknown origin. Three male patients on separate occasions admitted to the teaching hospital with a febrile illness, generalized maculopapular rash (1) and eschar (1). Blood testing revealed increased transaminases and C-reactive protein. Peripheral blood was collected prior to empirical antibiotic therapy and processed for NGS within 48 hours. DNA reads were searched against microbial genomic databases; the best mapped to *Rickettsia conorii* (patient 1), *Coxiella burnetii* (patient 2) and *Orientia tsutsugamushi* (OT, patient 3). Based on the NGS results all three patients received doxycycline with quick symptom resolution in all cases. NGS diagnosis was confirmed based on seroconversion to *R. conorii* and *C. burnetii* in patients 1 and 2, respectively, and by positive 56-kDa gene OT PCR of eschar (patient 3). Further in-depth analysis of individual sequence reads permitted identification of *R. conorii indica* as the etiological agent in case 1, and *Orientia tsutsugamushi* Shandong-XDM2-type in case 3, while *Coxiella burnetii* appeared to be a novel genotype.

Conclusion: Use of NGS technology enabled specific identification of three pathogens causing non-specific febrile illnesses. Quick etiological diagnosis permitted timely correction of patient management plans and their rapid recovery from these potentially fatal diseases. Other work is needed to validate and standardize this approach to diagnosis of rickettsial diseases.

UK outbreaks of Q fever: strain characterisation and assessment of aerosol survival.

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Coxiella burnetii, the causative agent of Q fever, is highly infectious by the aerosol route. A source of human outbreaks is from exposure to the birthing products of infected domesticated livestock, as large numbers of bacteria may be aerosolised into the environment during delivery. *C. burnetii* is then capable of spreading to human inhabitants surrounding the farm by windborne distribution or through exposure to contaminated waste materials such as bedding. *C. burnetii* is endemic within the UK with 904 human cases of acute Q fever being recorded between 2000 and 2015 in England and Wales. This study aimed to increase knowledge of the different genotypes of *C. burnetii* circulating within Great Britain and analysed 51 *C. burnetii*-containing abortion samples. These samples were obtained from farmed ruminants (sheep, goat and cattle) between 2013 and 2018. The samples were assigned to genomic groups II and III, with the genotype associated with the Netherlands outbreak (MST33) found in tissues originating from both goats and sheep. To better understand the ability of *C. burnetii* to spread from an infected farm we studied its survival within a contained dynamic aerosol for two hours. These data can contribute to informing the downwind hazard risk posed by *C. burnetii* should an outbreak occur at a farm.

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Rickettsia conorii disrupts inter-microvascular endothelial cell tight junction via activating NLRP3 inflammasome

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Rickettsia spp. cause life-threatening diseases in humans. The fundamental pathological changes in fatal rickettsial diseases are disrupted endothelial barrier and increased microvascular permeability, for which the mechanisms have not been fully understood yet. In the present study, we investigated the molecular basis of endothelial hyperpermeability caused by tick-borne rickettsiae. By confocal immunofluorescence microscopy and immunoblotting, we found that *Rickettsia conorii* infection disrupted the continuous immunofluorescence expression of the inter-endothelial tight junction protein, ZO-1, in infected microvascular endothelial monolayers, accompanied by diminished total expression levels of ZO-1. Interestingly, *R. conorii* and *R. parkeri* activated NLRP3 inflammasome in endothelial cells, as evidenced by increased expression levels of NLRP3 and cleaved caspase-1 and/or IL-1β in the cell lysates of infected endothelial cells. Furthermore, specific inhibition of NLRP3 by MCC950 significantly suppressed the activation and cleavage of caspase-1 induced by *R. conorii* in endothelial cells, which further prevented the disruption of inter-endothelial junctions and reduction of ZO-1 expression. Pharmaceutical inhibition of NLRP3 inflammasome did not significantly alter the concentrations of *R. conorii* in endothelial cells or viability of the cells compared to untreated controls. Taken together, our results suggest that R. conorii caused microvascular endothelial hyperpermeability likely via diminishing and interrupting the junctional protein ZO-1 through activating NLRP3 inflammasome. These data not only highlight the potential of ZO-1 as the biomarker for Rickettsia-induced microvascular injury but also provide insight into targeting NLRP3 inflammasome as a potentially adjunctive therapeutic for severe rickettsioses.

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An antigen-based diagnostic lateral-flow-assay for acute spotted fever rickettsioses

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Tick-borne spotted fever rickettsioses (SFR) can be life-threatening, with most mortality resulting from the lack of a timely and specific diagnostic assay. We previously reported the identification and detection of a diagnostic biomarker, the putative N-acetylmuramoyl-l-alanine amidase RC0497, by mass spectrometry (MS) both in experimentally infected animals and in patients. Here, we aim to develop an RC0497-in endemic areas. By stable isotope dilution (SID)-parallel reaction monitoring (PRM)-MS, we based assay which can be used for early diagnosis of SFR found that the concentrations of RC0497 in sera ranged from 0.1 to 1.1 ng/ml at the early stage of disease in *R. rickettsii*-infected guinea pigs and *R. conorii*-infected mice. By using mouse monoclonal and rabbit polyclonal antibodies against recombinant RC0497 as capture and detection antibodies, respectively, we developed a sensitive chemiluminescent sandwich ELISA which successfully detected the analyte RC0497 in sera of *R. rickettsii*-infected guinea pigs. Rabbit polyclonal antibody (RPAb) 721, but not mouse monoclonal antibody 9G1-7, showed reactivities against both *R. conorii* and *R. rickettsii* by indirect immunofluorescence assay (IFA). Using europium chelate particle reporters, we developed a prototype lateral-flow-assay (LFA) and observed a preliminary limit of detection of 0.5 ng/ml for RC0497 protein spiked into serum. This ongoing work, for the first time, demonstrated a low-cost and easy-to-use platform offering a timely point of care diagnostic assay for severe SFR.

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A single dose immunization of live-attenuated *R. parkeri* mutant confers complete protection against two fatal rickettsioses in mice

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Although with many years of attempt, no licensed vaccine is currently available for fatal rickettsial infections in humans. In the present study, we evaluated the safety and protective efficacy of live-attenuated vaccine candidate against fatal rickettsioses in mice. The genome of low-virulent rickettsiae, *R. parkeri*, was engineered by inserting a modified pLoxHimar transposon into the gene (RPATATE_0245) encoding a phage integrase protein. *Rickettsia parkeri* mutant RPATATE_0245::pLoxHimar (named 3A2) expressed mCherry fluorescence, resistance to spectinomycin, and attenuated virulence without a defectiveness in growth. A single dose immunization of *R. parkeri* 3A2 conferred complete protection against both fatal *R. parkeri* and *R. conorii* rickettsioses in mice. Compared to mock-immunized controls, mice immunized with *R. parkeri* 3A2 developed robust and durable IgG antibodies reactive against several rickettsial species, including *R. parkeri*, *R. conorii* and *R. rickettsii*. Furthermore, *R. parkeri* 3A2-conferred protection against two lethal rickettsioses in mice was also associated with an elevated percentage of IFN-γ-producing memory $CD4^+$ and $CD8^+$ T cells determined by intracellular flow cytometric analysis in response to *ex vivo* stimulation with *R. parkeri* and *R. conorii* compared to controls. In summary, our proof-of-concept study highlights the potential of *R. parkeri* mutants as a live-attenuated and multivalent vaccine platform in response to emergence of life-threatening spotted fever rickettsioses.

Ectoparasite profile and *Rickettsia* burden in small mammals collected in central Pennsylvania

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Small mammals can act as reservoirs of several tick-borne diseases, yet little is known about the role vertebrates play in sustaining these diseases within the state of Pennsylvania. Small mammals (*n=63*), including *Peromyscus leucopus*, *Tamias striatus*, and *Blarina brevicauda*, were trapped at three different locations in South Central Pennsylvania from June 2019 to September 2019. Upon capture, the mammals were identified to the species level and sexed. Cardiac puncture was performed on anesthetized animals to collect blood samples, ectoparasites were removed visually/ manually from the bodies of the small mammals prior to performing an ectoparasite wash, and each small mammal was necropsied. DNA was extracted from all tissues (liver, lung, heart, kidney, spleen), and the genus-specific Rick17b qPCR assay was used to test for the presence of rickettsiae in the samples. Five samples (*n= 367*) tested positive for *Rickettsia*. A total of 523 ectoparasites were identified to the genus or species level. The most common ectoparasite recovered was *Ixodes scapularis* (larvae); other ectoparasites collected included *Listophorus* sp., Dermanyssidae, *Peromyscopsylla* sp., *Dermacentor variabilis*, *Cuterebra angustifrons*, and *Polyplax* sp. Because Pennsylvania offers a variety of outdoor activities (hunting, hiking, camping, etc.), it is important to understand the environmental enzootic maintenance of vector-borne microbes in order to better understand the potential spread of human and veterinary diseases and to assess the risk of humans as accidental hosts for the disease-causing vectors.

Altered lethality associated with cytokine (IFNγ, TNF, and IL2) depletion in a non-lethal murine model of *Orientia tsutsugamushi* infection (compared to lethal and sublethal models)

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Interstrain variations among *Orientia tsutsugamushi* are associated with virulence disparities in murine models of scrub typhus. We previously compared the host response(s) in lethal (Karp, intraperitoneal (IP)), sublethal (Gilliam, IP) and non-lethal (Karp intradermal (ID), Woods, IP/ID, Gilliam ID) murine challenge models of *O. tsutsugamushi.* Multicolor flow cytometry analysis of CD4+ T-cell populations (including intracellular production of IFNγ, TNF, and IL2) revealed significant inter- and intra-strain increases in CD4+ IFNγ-IL2-CD4 and IFNγ-IL2-producing cells in the lethal and sublethal models. Following the assessment of multifunctional T-cell populations, which revealed the cytokine signatures associated with murine lethality/protection, we proceeded to further investigate the role of the Th1 cytokines (IFNγ, TNF, and IL2) via depletion experiments. Mice were treated with an anti-cytokine cocktail (mixture of antibodies against IFNγ, IL2, and TNF), or with isotype controls, subsequently challenged with *O. tsutsugamushi* Karp (either intraperitoneally or intradermally), and monitored for survival and immune response for 21 days following challenge. Historically, we have observed that mice challenged IP with *O. tsutsugamushi* Karp demonstrate low survival rates (0-10%), while ID challenged mice display 100% survival. In the instance of cytokine depletion, historically similar survival rates were observed for the mice that were lethally challenged (IP). However, upon ID challenge, we also observed 100% lethality in the mice treated with anti-cytokine cocktails, suggesting that the cytokines (IFNγ, IL2 and TNF) either in combination or singly, have an important role in the infection process. Analysis of the bacterial loads and seroconversion dynamics associated with the depletion experiments are currently in progress, as well as an expanded analysis of bacterial trafficking associated with the ID murine model.

Bovine Anaplasmosis prevalence assessed in beef cattle from diverse geographic regions of California

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Anaplasmosis in cattle resulting from *Anaplasma marginale* infections is a major economically important disease in the USA and many parts of the world. Clinical signs in infected animals vary from high fever, anemia, icterus, weight loss, lowered milk and meat production, lethargy, and death. The pathogen also persists for the life of an animal and the infected hosts serve as reservoirs. This tick-transmitted pathogen is also mechanically transmitted to naïve animals through fomites and biting flies, thus leading to multiple modes of disease spread in a herd. We evaluated bovine anaplasmosis prevalence in California beef cattle. A convenience sample of 9-38 cattle 2 years or older per herd representing 18 California counties were tested for infection status by competitive enzyme-linked immunosorbent assay (cELISA) and by quantitative TaqMan probe-based PCR targeting 16S rDNA gene. Infection prevalence by cELISA was 53%, in comparison to 44% by qPCR. About 83% of all cELISA positives also tested positives by qPCR. Nearly 100% of the qPCR positive animals were positive by cELISA. Several herds tested 100% positive, while other herds were 100% negative for the disease. There was no obvious geographic separation of counties with high infection prevalence compared to low to no infections. This study demonstrates that bovine anaplasmosis is widespread across the state of California with varying prevalence levels, possibly due to differences in herd management practices and/or differences with respect to vector habitat. Our study conclusions are consistent with prior reports documenting high bovine anaplasmosis prevalence in US cattle. This study highlights the importance of developing effective methods of control and prevention of bovine anaplasmosis, such as developing a vaccine conferring the protection against this economically important disease. (The research received support from the Russell L. Rustici Rangeland and Cattle Research Endowment at University of California, Davis and Livestock Memorial Research Fund, California Cattlemen's Association, Sacramento, CA. This work was also supported by the PHS grant # AI152418 from the NIAID, NIH, USA.)

IgG and IgM antibody Reactivity with individual spotted fever *Rickettsia* antigens

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Using a series of purified Spotted Fever Group (SFG) outer-membrane proteins we compared reactivity across this profile with separate IgG and IgM ELISA panels. Sera for these panels was submitted by our distributor in Switzerland, hence the European source for these species and several in which the source appeared to be vacations along the eastern coast of the Americas. Examples are shown in the IgG panels that the IgG responses appear to be species-specific, showing narrow spikes of reactivity to one or more different species. In contrast, the IgM panels were very broadly reactive against this entire panel suggesting different epitopes involved in the initial host responses. Both sets of panels utilized purified heteroduplexes of Omp B and Omp A, the result of a very high-affinity binding between these two protein components of the SFG s-layer. This heteroduplex binding was described on our poster at the Squaw Valley ASR Meeting. The lipopolysaccharide component was removed by a series of extractions.

Targeted mutagenesis in *Anaplasma marginale* to define virulence and vaccine dvelopment against bovine Anaplasmosis

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Tick-borne *Anaplasma* species are obligate, intracellular, bacterial pathogens that cause important diseases globally in people, agricultural animals, and dogs. Targeted mutagenesis methods are yet to be developed to define genes essential for these pathogens. In addition, vaccines conferring protection against diseases caused by *Anaplasma* species are not available. In this study, we describe a targeted mutagenesis method for deletion of the phage head-to-tail connector protein (*PHTCP*) gene in *Anaplasma marginale*. The mutant *A. marginale* did not cause disease and exhibited attenuated growth in its natural host (cattle). We then assessed its ability to confer protection against wild-type *A. marginale* infection challenge. Additionally, we compared vaccine protection with the mutant to that of whole cell *A. marginale* inactivated antigens as a vaccine (WCAV) candidate. Upon infection challenge, non-vaccinated control animals developed severe disease, with an average drop in packed cell volume (PCV) to 57% between days 26-31 post infection, an 11% peak in erythrocytic infection, and apparent anisocytosis. Conversely, following virulent infection challenge, all animals receiving the live mutant did not develop clinical signs or anemia, or erythrocyte infection. In contrast, the WCAV vaccinees developed similar disease as the non-vaccinees following virulent *A. marginale* infection, though the peak erythrocyte infection reduced to 6% and the PCV dropped 43%. This is the first study describing targeted mutagenesis and its application in determining *in vivo* virulence and vaccine development for an *Anaplasma* species pathogen. This study will pave the way for similar research in related *Anaplasma* pathogens impacting multiple hosts. (This work was supported by the PHS grant #s AI070908 and AI152418 from the NIAID, NIH, USA. The research also received support from the Russell L. Rustici Rangeland and Cattle Research Endowment at University of California, Davis and Livestock Memorial Research Fund, California Cattlemen's Association, Sacramento, CA.)

Endothelial exosome plays functional role during rickettsial infection

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Spotted fever group rickettsioses (SFRs) are devastating human infections. Vascular endothelial cells (ECs) are the primary targets of infection. Edema resulting from EC barrier dysfunction occurs in the brain and lungs in most cases of lethal SFR, but the underlying mechanisms remain unclear. The aim of the study is to explore the potential role of *Rickettsia* (*R*)-infected, EC-derived exosomes (Exos) during infection. Using size-exclusion chromatography (SEC), we purified Exos from conditioned, filtered, bacteria-free media collected from *R*-infected human umbilical vein ECs (HUVECs) (*R*-ECExos) and plasma of *R*-infected mice (*R*-plsExos). We observed that rickettsial infection increases the release of heterogeneous plsExos, but endothelial exosomal size, morphology, and production were not significantly altered following infection. Compared to normal plsExos and ECExos, both *R*-plsExos and *R*-ECExos induced dysfunction of recipient normal brain microvascular ECs (BMECs). The effect of *R*-plsExos on mouse recipient BMECbarrier function is dose-dependent. The effect of *R*-ECExos on human recipient BMEC barrier function is dependent on exosomal RNA cargo. Next-generation sequencing analysis and stem- loop quantitative reverse transcription PCR (RT-qPCR) validation revealed that *R* infection triggered the selective enrichment of vasculopathy-related microRNAs. Single living cell fluidic AFM coupling conventional cell biological studies demonstrated that endothelial

exosomal mir- 23a and mir-30b target the endothelial barrier and induce disruptions of both tight junctions and adherens junctions, which are the basic junctional components of the EC barrier, in the recipient EC. Our results shed light on the role and mechanism of exosomal microRNAs in the pathogenesis of R. infection and other EC-targeting pathogens.

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Multiple *Erhlichia chaffeensis* genes critical for its persistent infection in a vertebrate host are nonessential for its growth in the tick vector, *Amblyomma americanum*

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Ehrlichia chaffeensis, a tick-transmitted obligate intracellular rickettsial agent, causes infections in people, dogs, and several other vertebrates. We recently reported the generation and mapping of 55 new random mutations and used the mutant organisms to define the critical nature of genes for *E. chaffeensis* growth in the physiologically relevant canine host. The study aided the identification of many proteins as critical for the pathogen's persistent *in vivo* growth (Wang *et al.* 2020, *Infect. Immun.*). Using our recently established needle-infection inoculation method, we tested the infectivity of 45 of those mutants to naïve nymphal *Amblyomma americanum* ticks and their persistence during molting to the adult stage. Virtually all genes identified as essential for the *E. chaffeensis* growth in the canine host were found to be nonessential in the tick vector, except for three genes. Further, we identified mutations in two genes having no impact in the bacterial persistence in the canine host were found as essential for *E. chaffeensis* persistence in the tick host. The study demonstrates that *E. chaffeensis* requires many functional genes involved in biotin biosynthesis, protein and fatty acid biosynthesis, DNA repair, electron transfer, and a component of a multidrug resistance (MDR) efflux pump for its growth in the vertebrate host, while the genes were not essential for its growth in the tick vector. Similarly, three genes encoding for immunodominant membrane proteins; two 28 kDa outer membrane proteins (P28-1 and OMP-1V) and a 120 kDa immunodominant surface protein were required only to support *E. chaffeensis* replication within the vertebrate host. The study reveals that the pathogen adaptation mechanisms are distinct for *E. chaffeensis* for its tick vector compared to vertebrate host. While we also identified three genomic sites as essential for the *E. chaffeensis* adaptation for both tick and vertebrate hosts, their functional significance remains to be defined, as all the three mutations are within genes coding for hypothetical proteins. These data serve as a major resource for advancing understanding of how *E. chaffeensis* and possibly other related *Anaplasmataceae* family pathogens adopt to two distinct host environments: vertebrate hosts and ticks. (This work was supported by the PHS grant #s AI070908 and AI152418 from the NIAID, NIH, USA.)

Investigating the prophylactic and therapeutic potential of doxycycline using an A/J mouse aerosol model of Q-fever

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Outbreaks of Q fever have previously been reported in military personnel worldwide and there is continued requirement to minimise the likelihood of infection and reduce the severity of the disease. *C. burnetii* can persist within intracellular vacuoles requiring protracted treatment of chronic infection with a risk of relapse. We aim to improve the therapeutic options available by understanding the efficacy of existing therapeutics, optimise treatment windows and investigate co- therapies with licenced drugs. Doxycycline is the current standard treatment for Q fever, however therapy can be complex, lengthy and cause side effects. Doxycycline has also been suggested to prevent Rickettsial infections in travellers, although there is limited evidence to support the use of doxycycline as a prophylactic. Here, we use an A/J mouse aerosol model of Q fever to further evaluate the efficacy of doxycycline using alternative drug formulations and treatment regimes. We evaluated tolerability and efficacy of different preparations of doxycycline (hyclate or monohydrate), either as a chemoprophylaxis or as a post infection therapeutic. In our model, chemoprophylaxis successfully reduced clinical signs, however mice were still colonised at the end of the study. Yet, mice that received treatment upon the on-set of symptoms had significantly reduced bacterial load within their tissues. Therefore, chemoprophylaxis may not improve long term outcomes and may lead to a protracted incubation period, relapse and increased dissemination of bacteria once treatment has stopped. This suggests that timely diagnosis and early treatment, upon display of symptoms, is of therapeutic benefit. Notably, none of the therapeutic regimens provided sterile immunity at treatment group level. Given bacteriostatic nature of doxycycline and the emergence of doxycycline resistance, we are expanding our investigations to assess the feasibility of co-therapies using other licensed drugs. We have recently developed methodologies for the high throughput screening of an FDA approved compound library both in laboratory media and in a THP-1 model of infection. Primary screens have identified a number of hits to take forward to synergy screens with doxycycline.

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Advances in laboratory diagnosis of rickettsial diseases at the acute stage of illness

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Introduction: *Rickettsia* are obligate intracellular bacteria and have low levels of bacteria circulating in blood during early infection. During this early stage, symptoms of rickettsial disease appear as nonspecific and may include fever, headache, and malaise, with or without the observation of a rash or eschar. Clinical diagnosis also requires information on arthropod exposure and travel history up to 2 weeks prior to symptom onset. Any inaccuracies in patient recollection may delay disease suspicion and the collection of an appropriate sample. These factors may delay diagnostic laboratory testing and compromise an accurate diagnosis. **Methods:** We have examined sample stability in 3 common blood collection tubes, methods in cell culture amplification, and molecular detection methods to advance laboratory diagnosis of Rickettsial diseases. **Results:** 1) Acid citrate dextrose solution A (ACDA) blood collection tubes preserves PCR detectable *Rickettsia* DNA and ribosomal RNA targets more effectively over 7 days than the currently most commonly utilized collection tube, ethylene-diamine-tetra-acetic acid (EDTA). 2) Cell culture amplification from unprocessed patient blood may amplify spotted fever group *Rickettsia* within 3 days of inoculation, for PCR detection. 3) Ribosomal RNA, *Rickettsia spp*. detection lowers the limit-of-detection of real-time PCR assays by ~100x, from 1,800 genomic equivalents/mL of blood to 20 genomic equivalents/mL of blood. **Conclusions:** The challenges of laboratory diagnosis of *Rickettsia* spp. in acute blood samples have been investigated and methods have been defined to understand and overcome issues involving sample stability and low bacteremia. These findings represent important steps in the pursuit of more accurate diagnosis of Rickettsial diseases. Further studies to understand clinical sensitivity and utility are ongoing.

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Recognition of Vita-PAMPs in *Orientia tsutsugamushi* by endosomal RNA receptors

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Orientia tsutsugamushi enters the cell through the endocytic pathway, but soon after escapes the endosome to replicate in the cytoplasm. Live *Orientia* are potent inducers of pro-inflammatory cytokines (TNF-a, IL-6) and type I interferons (IFNs). We and others observed that heat inactivation of *Orientia* altered its capacity to induce certain cytokines, suggesting a specific recognition mechanism for live in contrast to dead bacteria. In mouse bone marrow-derived dendritic cells (BMDC) infected with *Orientia*, inhibition of endosome acidification by chloroquine or bafilomycin A abrogated the induction of IFN-b and TNF-a, suggesting that endosomal maturation is required for immune activation. Reduced cytokine induction upon infection and stimulation of BMDC deficient for the adapter protein MyD88 suggested a Toll-like receptor (TLR) involved in this recognition. BMDC from triple-knockout mice lacking the TLRs 3, 7 and 9 showed reduced transcription of IFN-b and TNF-a, upon both infection and stimulation with heat-inactivated *Orientia*, compared to the wildtype. Using single TLR-deficient BMDC, we demonstrated that TLR7, but not TLR3 or 9, was required for induction of IFN-b and TNF-a mRNA upon infection with live *Orientia*. Contrarily, heat-inactivated *Orientia* were recognized by TLR3, but not TLR7, suggesting differential receptor usage for recognition of RNA from *Orientia*. Residual RNA-dependent stimulation in TLR3-deficient compared to MyD88-deficient BMDC could be explained by usage of TLR13 by heat-inactivated *Orientia*. Investigations in THP1 macrophages showed that in human cells, TLR8 is responsible for induction of TNF-a by viable *Orientia*. Altogether, our study reveals RNA recognition as the major mechanism of early innate inflammation induced by *Orientia* in mice and humans, with murine TLR7 and human TLR8 recognizing live organisms, and murine TLR3 and TLR13 involved in RNA recognition of heat-inactivated bacteria. The employment of TLRs for endosomal RNA recognition in humans and mice thus depends on the viability of *Orientia*.

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Whole-genome sequencing of *R. amblyommatis* isolated from *A. americanum* in Long Island reveals unique genetic traits and phenotypes associated with mild rickettsioses

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Ticks transmit multiple human pathogens of public health importance. Recent environmental changes have fostered the expansion of invasive tick species into new geographical areas and the emergence of tick-borne diseases. Over the last decades, *Amblyomma americanum* (lone star ticks) has recolonized, thrived, and expanded into the upper midwestern and northeastern United States. Lone star ticks feed on large mammals, especially white-tailed deer, and display non-discriminative and aggressive biting behaviors, increasing the possibility of tick bites and transmitting tick-borne pathogens. Interestingly, the resurgence and expansion of lone star ticks are linked to increased cases of mild spotted fever rickettsioses. Prior studies document that a large proportion of lone star ticks are infected with *Rickettsia amblyommatis* and may transmit *R. amblyommatis* during blood-feeding. Previous *in vivo* infection studies reported that *R. amblyommatis* is capable of causing mild clinical symptoms in guinea pigs and mice. However, the pathogenic capacity of *R. amblyommatis* in humans remains to be determined. Further, there is a significant gap in knowledge on *R. amblyommatis* in lone star ticks present in newly established geographical areas. Here, we isolated *R. amblyommatis* from lone star ticks in Long Island, New York, performed whole-genome sequencing analysis to determine unique genetic traits, characterized *in vitro* replication phenotypes, and determined the presence of *R. amblyommatis*-specific antibody responses in patients. Our study implicates the potential genetic adaptation of *R. amblyommatis* in lone star ticks and corroborates the existing evidence that *R. amblyommatis* may cause mild rickettsioses.

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Importazole, a specific inhibitor of importin-dependent nuclear transport, suppresses growth of *Anaplasma phagocytophilum* in HL-60 cells by preventing AnkA nuclear accumulation.

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Anaplasma phagocytophilum (AP) is a gram-negative obligate, tick-borne bacterium of neutrophils that causes human granulocytic anaplasmosis. Ankyrin A (AnkA), an ankyrin repeat protein essential for bacterial growth is delivered into the host cytoplasm by a type IV secretion system. After injection, AnkA translocates into the nucleus, binds to host DNA and reprograms epigenetics of the host cell to promote bacterial survival and growth. In this study, we utilized a small molecule importazole, a specific inhibitor of the importin-dependent nuclear transport system, to investigate its effects on AP growth inside the infected HL-60 cells. By using qPCR, we found that infected cells had 175 $\pm$ 75 bacteria per cell (mean $\pm$ SEM) after 48-72 hours post infection and showed significant decrease in host cell viability from 91+ 5% to 63 $\pm$ 3%, $p < 0.01$, measured by trypan blue exclusion method and automatic viability cell counter. In contrast, we found that incubation with 10 μM importazole strongly suppressed AP growth, to 15 $\pm$ 7 bacteria per cell, and significantly improved a viability of the infected cells, from 63% to 79+2% ($p < 0.05$). Furthermore, by using fluorescent microscopy-based colocalization analysis and cell transfection with eGFP-labeled AnkA plasmid constructs we also found that importazole significantly reduced nuclear localization of the AnkA in the model transfected HEK293 cells. In addition, when the cells were loaded with GTPγS, a non-hydrolysable analog of GTP that inhibits GTP-dependent cellular processes, including RanGTP-dependent nuclear protein transport, we observed a similar inhibitory effect on AP growth in the infected HL-60 cells and on the nuclear localization of eGFP-AnkA in the transfected HEK293 cells. These data show that AP infection and growth depend on AnkA translocation into the host nucleus through importin/RanGTP-dependent nuclear transport system.

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Inhibition of *Ehrlichia chaffeensis* infection by cell-permeable bicyclic peptides that bind ehrlichial type IV secretion effector Etf-1

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Ehrlichia chaffeensis is an obligatory intracellular bacterium that causes human monocytic ehrlichiosis (HME), an emerging life-threatening infectious disease worldwide. Infection with obligatory intracellular bacteria is difficult to treat as intracellular targets and delivery methods of therapeutics are not well understood. *Ehrlichia* translocated factor-1 (Etf-1), a type IV secretion system effector, is essential for *Ehrlichia* infection. In this study, we screened a synthetic library of cell-permeable bicyclic peptides, which consists of an ensemble of random peptide sequences in the first ring and a cell-permeable peptide in the second ring, for Etf-1 binding. Screening of over 320,000 bicyclic peptides and optimization of selected Etf-1-binding peptides identified over 40 peptides, which can bind to Etf-1 at high affinity (K_D ranges from 1 and 10 μM) with efficient mammalian cell cytosolic uptake and low cytotoxicity. Treatment of *E. chaffeensis*-infected THP-1 cells showed that several peptides, especially B7, C8, 3-131-5, 3-133-3, and 3-133-8, significantly inhibited *Ehrlichia* infection. Etf-1 was shown to have two intracellular localizations and functions:1) Etf-1 localizes to mitochondria and blocks host cell apoptosis, and 2) Etf-1 directly binds to Beclin 1 (ATG6) at high affinity to induce cellular autophagy and fuses with *E. chaffeensis* inclusions, which are critical for delivering host-cell cytoplasmic nutrients into ehrlichial inclusions. B7 did not inhibit Etf-1 mitochondrial localization. However, B7 and its derivatives inhibited the binding of Etf-1 to Beclin-1 and Etf-1 localization to *E. chaffeensis*-inclusion membranes. The results not only affirm the critical role and mechanisms of Etf-1 functions for *E. chaffeensis* infection, but also demonstrate the feasibility of developing cell-permeable bicyclic peptides to advance mechanistic research and potential treatment of diseases caused by *Ehrlichia* and intracellular pathogens.

EcxR is a global gene regular which interacts with the promoter segments of multiple Ehrlichia chaffeensis genes transcribed by RNA polymeraseloenzyme containing the housekeeping or alternative sigma factors

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Ehrlichia chaffeensis is a tick-transmitted intraphagosomal bacterium causing infections in people, dogs, and various vertebrate hosts. The pathogen has a biphasic developmental cycle during its growth in vertebrate monocytes/macrophages and tick cells. Host-specific differences in the gene expression from many genes *chaffeensis* of *E.* are also well-documented. It is unclear how the organism regulates gene expression during its developmental cycle and for its adaptation to vertebrate and tick host cell environments. We recently mapped promoters of several genes of the pathogen recognized by RNA polymerase (RNAP) having its only two sigma factors: the housekeeping σ70 and the alternate σ32. In the current study, we performed experiments in five assessing predicted *E. chaffeensis* transcription regulators (EcxR, CtrA, MerR, HU and Tr1) for their possible roles influencing in the bacterial gene expression. Promoter segments of three genes each transcribed with the RNAP containing σ70 (HU, P28-Omp14 and P28-Omp19) and σ32 (ClpB, DnaK and GroES/L) were investigated by employing multiple independent molecular methods. We demonstrate that EcxR is a global gene binding regulator to all six gene promoters assessed and that its promoter-specific interactions result in the varying gene expression for the genes as assessed by the *E. coli* surrogate system lacking its native sigma factors and complemented with the expression of *E. chaffeensis* sigma factors. These data confirm prior reportsdemonstrating the global regulator function of EcxR and its homologs in other related *Anaplasmataceae* family organisms. This study also demonstrated that EcxR plays an important role in regulating the expression of shock heat proteins. (This work was supported by the PHS grant # AI070908 from the NIAID, NIH, USA.)

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Q fever post-vaccination hypersensitivity modeling reveals sex dimorphism and a less reactogenic vaccine candidate

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Delayed-type hypersensitivity (DTH) responses to microbial vaccines and related components are a major roadblock for widespread licensing of whole cell vaccines such as that of Q fever. Q fever is a zoonotic disease caused by the intracellular bacterium *Coxiella burnetii*. Guinea pigs have been used to model late phase granulomatous DTH responses following Q fever vaccination; however, the early phase DTH response has not been recapitulated in this model. To address this, we developed a murine post Q fever vaccination DTH model which robustly represents the early phase DTH response observed in humans. In this model, female C57Bl/6J mice displayed the most intense early-phase DTH responses following sensitization and elicitation compared to their male counterparts and other mouse strains. Immunologic responses were measured within the skin, draining lymph nodes, and serum following both sensitization and elicitation with Q fever whole cell vaccines (WCV). Local immunologic responses in the dermis were characterized by inflammation primarily involving neutrophils, macrophages, and T cells. Secondary lymphoid organ profiling revealed distinct immunological signatures following both sensitization and elicitation with a sex-based dichotomy in T cell phenotypes and antigen presenting cell numbers. Next, employing the guinea pig post-vaccination DTH model, we screened a genetically modified *C. burnetii* Dugway Δ*dot/icm* WCV, which demonstrated reduced reactogenicity and less severe granulomatous DTH responses. The murine early-phase DTH model will be used for future vaccine screening and immunologic interrogation. Beyond providing a post-Q fever vaccination DTH model that recapitulates early-phase DTH events, these data suggest that sex is a primary factor influencing the magnitude and composition of the ensuing response and that genetically modified WCVs are viable vaccine candidates for Q fever.

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Ehrlichia canis immunome is dominated by hypothetical proteins and conformation-dependent antibody epitopes

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A small group of *Ehrlichia* immunoreactive proteins, including the well-characterized tandem repeat proteins (TRPs) that have linear antibody epitopes, has previously been defined by conventional immunoblotting, but it likely represents an incomplete immunome of these pathogens. Recently, we implemented a high-throughput antigen discovery strategy that combines genomics, bioinformatics, cell-free protein expression, and immunoscreening approaches to rapidly identify new immunoreactive proteins from *E. canis*. The entire ORFeome of *E. canis* were analyzed by the sequence-based prediction tool ANTIGENpro, and we identified 350 proteins as highly antigenic (antigenicity score > 0.6). The immunoreactivity of top 350 proteins and all other hypothetical proteins present in the ORFeome (n=412) was investigated. In total, 77 *E. canis* proteins reacted with sera from naturally *E. canis*-infected dogs. A subset of 17 *E. canis* proteins were identified as immunodominant, comparable to defined major immunoreactive protein TRP19. Most immunodominant proteins were classified as hypothetical, and the antibody epitopes exhibited complete or partial conformation dependence. The majority (9/17) of these proteins were predicted to be secreted effectors, and 8 proteins contained transmembrane domains. This study substantially expands the immunomes of *E. canis* and reveals the importance of largely unexplored conformation-dependent epitopes as targets of anti-*Ehrlichia* immune responses. These findings advance efforts to define the molecular features of protective proteins and improve prospects for effective vaccines for the ehrlichioses.

Identification of transposon insertion mutants in pathogenic SFG *Rickettsia* species that are impaired in intracellular growth within mammalian phagocytic cells.

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Bacteria in the genus *Rickettsia* are arthropod-borne, obligate intracellular pathogens, causing a range of febrile diseases from spotted fevers to typhus. Spotted Fever group (SFG) *Rickettsia* pathogens include *R. conorii, R. rickettsia and R. parkeri,* which cause severe disease throughout the Mediterranean/European and American areas, respectively. *R. rickettsii,* specifically, is the causative agent of the most severe human spotted fever rickettsiosis (SFR), Rocky Mountain Spotted Fever (RMSF). RMSF has been in most of the continental United States (US) and recently severe strains have been found in Central and South America. According to the Center of Disease Control and Prevention, the number of SFR cases in the US has steadily increased since the early 2000s due in part by clinical misdiagnosis of febrile cases. Diagnostic techniques have improved, and this has coincided with an overall decrease in the fatality rate of SFR cases; however, the fatality rate for diagnosed RMSF has remained higher (5-10% of cases are fatal) and is as great as 20% if left untreated. Vascular endothelial cells are considered the major target cell of SFG rickettsial infections and have been a focus of study to elucidate molecular details governing *Rickettsia*-host cell interactions. Rickettsiae induce internalization into these cells, quickly escape from an endosomal-like compartments and replicate within host cell cytosol. However, in addition to parasitism of endothelial cells both *in vivo* and *in vitro*, our research team and others groups in the field of Rickettsiology have also demonstrated that pathogenic *Rickettsia* species invade into and replicate within other cell types, including lymphocytes, neutrophils, hepatocytes, monocytes and macrophages. In two recent published studies, our research team demonstrated that multiple recognized human pathogenic species of *Rickettsia*, including *R. conorii*, *R. rickettsii*, *R. parkeri*, *R. africae*, and *R. akari* can grow within target endothelial cells as well as within PMA-differentiated THP-1 cells as a model for macrophages. In contrast, *R. montanensis* and *R. bellii*, two *Rickettsia* species not associated with disease of humans, and *R. rickettsii* strain Iowa, an avirulent derivative of pathogenic *R. rickettsii*, could invade both endothelial cells and THP-1 macrophages, but proliferate only within endothelial cells in these studies. Further analysis revealed that recognized pathogenic *Rickettsia* species could grow within the cytosol of THP-1-derived macrophages and avoided localization with two different markers of lysosomal compartments, LAMP-2 and cathepsin D. The lack of *R. montanenesis* and *R. bellii* growth in macrophages was correlated with a significant co-localization with markers of endosomal and lysosomal compartments. These results demonstrated that closely related *Rickettsia* species can differ considerably in their abilities to proliferate within different cell types and suggest that this characteristic could affect its virulence potential.

However, the molecular mechanisms by which model pathogenic SFG Rickettsia species invade into phagocytic cells and establish a proliferative intracellular niche have not been elucidated.These results also led to the hypothesis that pathogenic *Rickettsia* species may possess uncharacterized factors that contribute to specific intracellular growth within macrophage-like cells in vitro. To initially test this hypothesis, we recently obtained a small panel of published transposon insertion mutants in *R. parkeri* strain Portsmouth and performed quantitative PCR (qPCR)-based growth assays on individual mutants in both endothelial (EAhy.926 cells) and macrophage-like THP-1 cells. Interestingly, all the tested mutants proliferated to levels within endothelial cells consistent with control non-transformed *R.* parkeri strain Portsmouth. However, at least one mutant was able to bind to and invade into THP-1 macrophages but failed to proliferate within these cells. We are currently investigating the mechanism(s) by which this single transposon insertion leads to growth defects within phagocytic cells. Taken together, these results suggest that transposon insertions into single genes within pathogenic *Rickettsia* species can yield mutants that are defective for growth within subsets of target cells and may shed further insight into the mechanisms by which *Rickettsia* species interact with mammalian cells. In addition, the feasibility of performing random transposon mutagenesis in several other SFG *Rickettsia* species including *R. conorii* and *R. rickettsii* suggests that this methodology is a powerful tool to determine factors that contribute to intracellular growth and may reveal *bona fide* virulence determinants within this class of obligate intracellular bacteria.

A transposon screen to identify potential essential genes in *Coxiella burnetii*

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Coxiella burnetii is an intracellular pathogen responsible for causing Q fever in humans, a disease with varied presentations ranging from a mild flu-like sickness to a debilitating illness that can result in endocarditis. Current treatment is limited to extended periods of antibiotic treatment, with no licenced vaccine available in the UK or US at present. Together, this presents a worrying situation which calls for improved antibiotic therapies and/or the production of a safe, effective vaccine. Transposon-directed insertion site sequencing (TraDIS) is a negative selection screen that has been applied to identify essential genes of many bacterial species under a range of biologically relevant conditions. Application of this technique to *C. burnetii* Nine Mile Phase II aims to provide information on *C. burnetii* genes fundamental for the growth and survival of this pathogen in axenic media, enabling further analysis of essential gene products as prospective drugable targets. To identify essential genes in a high-throughput manner, a transposon library containing > 10,000 unique transposon mutants was created, revealing 512 essential genes. These genes were further analysed *in silico* to investigate their conservation in the core genome of *C. burnetii* and their potential drugability.

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Investigation of genotype variations among *Orientia tsutsugamushi* human isolates as possible predictors of severe scrub typhus

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Orientia tsutsugamushi is known to cause highly variable disease presentations and mortality rates. The significant genetic diversity of *O. tsutsugamushi* may play a role in this variability, yet full genome, single nucleotide polymorphism (SNP), and gene polymorphism analyses of *O. tsutsugamushi* are limited. To investigate the relationship between disease severity and genetic diversity, 65 *O. tsutsugamushi* isolates were collected from patients in Laos between 2010- 2014. Disease severity was assessed, and patients were categorized to have severe disease if one of the following was present: Shock, Reduced consciousness, Meningitis/encephalitis, Jaundice, and/or Difficulty breathing. Twenty- nine patients met the case definition for severe disease. Whole genome sequencing was successful for 28 isolates: 14 from patients with severe disease. Sequences were mapped to the UT76, Thai isolate of *O. tsutsugamushi*. In addition, the outer membrane protein (*omp*) A, cell surface antigens (*sca*) A, C, and E, and ankyrin repeat proteins (*ank*) 1, 4, 6, and 9 genes were PCR amplified and sequenced for the 65 isolates and evaluated for possible polymorphisms associated with severe disease. High genetic diversity among isolates was seen, however, no significant SNPs were identified from the whole genome analyses. Gene fragments from *anks9, 4,* and *1* also showed no association with disease severity. Interestingly, several isolates from patients with severe disease contained the same unique polymorphisms in *ank6, scaA,* and *ompA.* Further, clades with high clustering of isolates

from patients with severe disease were seen for scaE, scaC, and scaA. Disease severity due to O. tsutsugamushi is complex and likely results from a variety of host and bacterial determinants. While this study did not identify any SNPs or definitive polymorphisms that play a role in disease severity, the results suggest that scaE, scaC, scaA, ompA and ank6 and possibly additional virulence factors warrant further investigation.

Role of lipid droplets and prostaglandinE2 in *Coxiella burnetii* intracellular growth

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The obligate intracellular bacterium *Coxiella burnetii* causes potentially fatal endocarditis several years after initial infection suggesting the bacterium's ability to persist long-term in the host. Our overall goal is to determine the mechanisms *Coxiella* employs for its long-term intracellular survival. While the bacterium initially infects alveolar macrophages, in endocarditis patients *Coxiella* is found in foamy macrophages rich in neutral lipid storage organelles called lipid droplets (LDs). Our previous studies show that *Coxiella* manipulates host LD metabolism via the Type 4 Secretion System (T4SS), a major virulence factor which secretes bacterial effector proteins into the host cell cytoplasm to manipulate cellular processes. Additionally, blocking LD breakdown almost completely inhibits bacterial growth suggesting that LD-derived lipids are critical for *Coxiella* intracellular survival. LD breakdown releases arachidonic acids, precursors for the lipid immune mediator prostaglandin E2 (PGE2) which promotes an immunosuppressive environment in alveolar macrophages. *We hypothesize that Coxiella manipulates host cell LD metabolism to promote a PGE2-mediated immunosuppressive environment and survive long-term in the host.* To test this, we quantified gene expression of PGE2 synthesis enzyme cyclooxygenase-2 (*cox-2*) in *Coxiella*-infected alveolar macrophages. Compared to uninfected cells, *cox-2* was upregulated in *Coxiella*-infected but not T4SS mutant-infected macrophages. ELISA showed *Coxiella*-dependent increase in PGE2 levels indicating that *Coxiella* T4SS actively manipulates *cox-2* expression resulting in increased PGE2 production. Further, blocking PGE2 production using FDA-approved COX-2 inhibitors significantly decreased *Coxiella* intracellular growth suggesting the importance of PGE2 during *Coxiella* infection. Ongoing studies are identifying the direct correlation between LDs and PGE2 production and the contribution of LDs to immunosuppression during *Coxiella* infection. Future studies will determine the potential of blocking PGE2 production as a supplemental therapy for *Coxiella* endocarditis.

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Anaplasma phagocytophilum: Getting around in ticks and humans

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In North America, the incidence of diseases caused by tick-borne pathogens has increased significantly. The black-legged tick is responsible for most of these, including an obligately intracellular pathogen, *Anaplasma phagocytophilum* (Ap), that causes human anaplasmosis, an acute febrile illness. These bacteria master a complex life cycle that requires moving among different host cells in the mammal following deposition into the tick bite site that eventually results in infection of neutrophil granulocytes (PMNs). Ticks ingesting infected blood acquire Ap, but their sojourn through tick tissues, how they survive molting and reach the salivary glands is incompletely known. Illuminating the natural history of tick-borne pathogens with information about gene expression and function is an important goal. Despite the small *Anaplasma* genome of ~1.5 Mbp, our studies have shown that the Ap genome is differentially transcribed in host and vector cells, with further differences in gene expression during extracellular phases, adhesion, invasion and intracellular replication. Genes specific to the Anaplasmataceae were the dominant group induced inside human cells, including several that are thought to encode effectors. Hypothetical genes were upregulated in human cells, especially PMNs, and are thus of particular interest for functional genomics studies. In cultured tick cell, dominant genes encoded membrane-associated or secreted hypothetical proteins. We selected 25 mutants from our library of random transposon mutants to test their ability to infect mice and ticks. All except two that disrupted a metalloendopeptidase, infected mice, but only half were acquired by ticks. Of interest, HGE_00035 encoding an isoprenoid synthesis protein, was detected in only 1 of 6 mice on day 7, and failed to become established in ticks. Given the importance of the isoprenoid pathway for bacterial virulence, and the reduced infectivity of this mutant, it may be a candidate for a live vaccine.

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A small non-coding RNA mediates transcript stability and expression of cytochrome bd-I ubiquinol oxidase subunit 1 in Rickettsia conorii

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Small regulatory RNAs are now well appreciated for their importance in post-transcriptional regulation of bacterial virulence and growth. Recently, we have shown the biogenesis and differential expression of several sRNAs in *Rickettsia conorii* during its interactions with the human host and arthropod vector and confirmed the binding of *Rickettsia conorii* sRNA *Rc*_sR42 to bicistronic cytochrome bd ubiquinol oxidase subunit I and II mRNA *in vitro*. However, the mechanism of regulation and the impact of sRNA binding on the stability of the *cydAB* bicistronic transcript and expression of *cydA* and *cydB* genes is not yet understood. In this study, we determined the expression dynamics of *Rc*_sR42 and its cognate target genes, *cydA* and *cydB*, in mouse lung and brain tissues during *R. conorii* infection *in vivo* and employed fluorescent and reporter assays to decode the role of sRNA in regulating cognate gene transcripts. Quantitative RT-PCR revealed significant changes in the expression of sRNA and its cognate target gene transcripts during *R. conorii* infection *in vivo* and greater abundance of these transcripts was observed during infection of the lungs compared to brain tissue. Interestingly, while *Rc*_sR42 and *cydA* exhibited similar pattern of change in their expression, indicating the influence of sRNA on mRNA target, the expression of *cydB* was independent of sRNA expression. Further, we constructed reporter plasmids of sRNA and *cydAB* bicistronic mRNA to decipher the role of sRNA on *cydA* and *cydB* expression. We observed increased expression of *cydA* in the presence of sRNA, but detected no change in *cydB* expression in the presence or absence of sRNA. In sum, our results demonstrate that the binding of *Rc*_sR42 is required for the regulation of *cydA* but not *cydB*. Further studies on understanding the influence of this interaction during *R. conorii* infection of mammalian host and tick vector are in progress.

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Proof-of-concept efficacy of doxycycline in an inhalational marmoset model of Q Fever

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Coxiella burnetii is a Gram negative intracellular bacterium and the causative agent of the zoonotic disease Q fever. Infection in humans usually occurs via the inhalation of aerosolised products from infected animals. Recommended treatment for acute Q fever is 100 mg of doxycycline for 14 days. However, in order to assess novel medical countermeasures, representative and predictive animal models are required. A marmoset model of Q fever has been developed and the efficacy of doxycycline was assessed to validate the model for future assessment of novel medical countermeasures. Common marmosets (*Callithrix jacchus*) were challenged with $3.2 \times 10^6 \pm 1.1 \times 10^6$ cfu of *C. burnetii* by the aerosol route. At 24 hours post-challenge animals received doxycycline or placebo by the oral route. Administration of doxycycline offered protection in terms of a reduction in weight loss, bacteraemia and reduced lung pathology and fever. Fever, IFN-g expression and bacteraemia were good indicators of the animal disease state, increasing during the peak disease in the placebo group. Overall, doxycycline therapy had a beneficial effect on the animals following administration 24 hours post-challenge, however disease was not totally ameliorated. Delivery of antibiotics as treatment (e.g. on the onset of fever) is likely to result in further amelioration of the disease, and warrants follow-on work.

This work was funded by a Project Agreement with DRDC, Canada: Chemical biological radiological (CBR) project arrangement no. uk-ca-a-15-02.

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Anaplasma marginle enters tick cells using clathrin and proteins of the Msp1 superfamily

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Anaplasma marginale, causes bovine anaplasmosis, a production-limiting, tick-borne disease of cattle found worldwide. Current methods to prevent this disease are inadequate. Importantly, because *A. marginale* is an obligate intracellular bacterium, blocking entry into host cells via neutralizing antibodies produced through immunization may serve as an essential component of a vaccination strategy. However, the molecules and mechanisms required for *A. marginale* entry into tick cells and bovine erythrocytes, the primary eukaryotic host cells, are unknown. To fill this knowledge gap, we determined that *A. marginale* enters tick cells via clathrin-mediated endocytosis but not macropinocytosis. This indicates that interference with receptor-ligand binding in the tick could reduce tick transmission. To identify the *A. marginale* surface proteins that mediate entry into tick cells and bovine erythrocytes, we constructed a phage display library consisting of the majority of *A. marginale* outer membrane proteins. Phage recovered from binding assays with either *Dermacentor andersoni* cells or bovine erythrocytes after multiple rounds of screening were identified using real-time PCR. Empty wells served as the negative control to identify phage that non-specifically bound plastic. The top adhesin candidates included OmpA, as predicted, members of the Msp1 family, and members of the type IV secretion system. Using recombinant proteins, we have determined that at least one member of the Msp1 family mediates entry into tick cells. This protein and potentially others will serve as important components of a multivalent vaccine to reduce tick transmission and bovine anaplasmosis.

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Detection and isolation of *Rickettsia tillamookensis* (Rickettsiales: Rickettsiaceae) from *Ixodes pacificus* (Acari: Ixodidae) from multiple regions of California

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The western black-legged tick (*Ixodes pacificus*) is the most frequently identified human-biting tick species in the western United States and the principal vector of at least three recognized bacterial pathogens of humans. A potentially pathogenic *Rickettsia* species, first described in 1978 and recently characterized as a novel transitional group agent designated as *Rickettsia tillamookensis*, also exists among populations of *I. pacificus*, although the distribution and frequency of this agent are poorly known. We evaluated DNA extracts from 348 host-seeking *I. pacificus* nymphs collected from 9 locations in five California counties, and from 916 *I. pacificus* adults collected from 24 locations in 13 counties, by using a real-time PCR designed specifically to detect DNA *R. tillamookensis.* DNA of *R. tillamookensis* was detected in 10 (2.9%) nymphs (95% CI: 1.6 - 5.2%) and 17 (1.9%) adults (95% CI: 1.2 - 3.0%) from 11 counties of northern California. Although site-specific infection rates varied greatly, frequencies of infection remained consistently low when aggregated by stage, sex, habitat type, or geographical region. Four novel isolates of *R. tillamookensis* were cultivated in Vero E6 cells from individual adult ticks collected from Alameda, Nevada, and Yolo counties. Four historical isolates, serotyped previously as “Tillamook-like” strains over 40 years ago, were revived from long-term storage in liquid nitrogen and confirmed subsequently by molecular methods as isolates of *R. tillamookensis*. The potential public health impact of *R. tillamookensis* requires further investigation.

Hacking the Tick: Identification and Characterization of a Tick-targeted Effector from *Anaplasma phagocytophilum*

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Anaplasma phagocytophilum must navigate the intracellular environments in both mammalian and tick cells in order to complete its lifecycle as a tick-transmitted pathogen. *A. phagocytophilum* manipulates the intracellular environment using a Type IV Secretion System (T4SS) to deliver effector molecules into the host cell cytosol. Few *A. phagocytophilum* T4SS translocated effectors have been characterized, with interactions focusing primarily on microbial manipulation of the mammalian host. Comparatively less is known about interactions with the tick and no tick-specific T4SS effectors have been identified. Here we identify a novel *A. phagocytophilum* protein HGE1_2492 that is important for growth and persistence in ticks, is T4SS translocatable, and manipulates the actin cytoskeleton. Transcriptional analysis found HGE1_2492 to be specifically expressed during ISE6 tick cell infection. A transposon insertion mutant disrupting HGE1_2492, HGE1_2492::himar1, disabled *A. phagocytophilum* growth in tick cells but had no impact on growth in mammalian cells. The HGE1_2492::himar1 mutant colonizes mice comparable to wild type but has a deficit in colonizing *Ixodes scapularis* larvae. We show HGE1_2492 is translocated in a T4SS dependent manner using a heterologous *Legionella pneumophila* CyaA reporter assay*.* Moreover, we identified that intrinsically unstructured regions of HGE1_2492 are sufficient for T4SS secretion. Ectopic expression of GFP-tagged HGE1_2492 localized with F-actin and altered cytoskeleton morphology. Truncation mutants identified separate regions of HGE1_2492 were responsible for actin co-localization, actin remodeling, and plasma membrane association. Altogether, this work represents the first investigation of an *A. phagocytophilum* T4SS effector that is important for colonization, and intracellular growth specifically in the arthropod vector.

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Evaluation of *Ehrlichia canis* diagnostic protein immunoreactivity and dual host expression

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Canine monocytic ehrlichiosis (CME) is caused by *Ehrlichia canis* (*E. canis*), a globally distributed pathogen that causes serious and life-threatening infections in dogs. Diagnosis of *E. canis* infection can be obtained by serologic evaluation using major immunoreactive proteins TRP19, TRP36 and TRP140 that have defined species-specific linear antibody epitopes. In this study we evaluated and compared multiple recently identified *E. canis* immunoreactive proteins with "gold standard" TRPs for serodiagnosis of CME. In addition, we examined the expression levels of TRPs with recently identified immunoreactive proteins in tick (ISE6) and mammalian (DH82) cell lines. Consistent with our previous studies, TRP19, TRP36 and TRP140 strongly reacted with antibodies in dogs naturally infected with *E. canis.* Moreover, several of the new major immunoreactive proteins reacted with antibodies in convalescent dog sera at similar or higher levels to the established TRPs by ELISA. To understand potential differences in diagnostic sensitivity for detection of antibodies generated early in infection, we also examined the expression levels of these immunoreactive proteins in *E. canis*-infected tick (ISE6) and mammalian (DH82) cells by immunofluorescence assay (IFA) and Western blot (WB) analysis. Several proteins (TRP78, 0073 and 0920) were predominately expressed in ISE6 cells, while others (TRP19, TRP36 and 0919) were predominately expressed in DH82 cells. These results demonstrate new candidates for *E. canis* immunodiagnosis with sensitivity comparable to TRPs, and reveal differential expression patterns that may translate to differences in sensitivity for detection of antibodies produced early in infection.

Examining the evolution of pathogenicity in *Coxiella* to identify novel therapeutic targets

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Pathogenicity of *Coxiella burnetii* is contingent upon it replicating within a lysosome-derived, acidic intracellular vacuole. However, the metabolic processes that underpin this unique ability are largely unknown. This lack of knowledge has hindered the development of better therapies against acute and chronic Q fever caused by *C. burnetii*. We utilized an evolutionary genomics approach to try and identify metabolic processes that contribute to *C. burnetii*'s intracellular growth and virulence. To this end, we sequenced the genome of the pathogen's closest known relative — an endosymbiont in the soft tick *Ornithodoros amblus* (CLEOA) — and by comparing it to the genome of *C. burnetii*, identified pathogen-specific metabolic pathways. In addition, by analyzing the genomes of all members of the order Legionellales (*Aquicella*, *Berkiella*, *Coxiella*, *Diplorickettsia*, *Legionella*, *Rickettsiella*) we show that the pathogenic capacity of *C. burnetii* is an ancestral trait, but the Q fever agent has gained several genes via horizontal gene transfer (HGT) that probably augment its virulence. By identifying metabolic pathways in *C. burnetii* that contain genes that are both HGT-derived and pathogen-specific we have delineated several physiological processes that are likely critical to *C. burnetii*'s intracellular growth and pathogenicity. Heme biosynthesis is one such process, and by inhibiting an enzyme involved in heme production or by blocking the incorporation of heme into proteins, we were able to significantly reduce the intracellular growth of *C. burnetii*. In sum, our data show that using an evolutionary lens to uncover metabolites essential to *C. burnetii*'s pathogenicity is a promising strategy to developing new treatment strategies.

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The Coxiella effector CvpG modulates fusion properties of the *Coxiella* containing vacuole

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Coxiella burnetii secretes at least 150 effector proteins into the eukaryotic host cell, controlling the formation and maturation of the large, fusogenic Coxiella Containing Vacuole (CCV). Six effectors, termed Coxiella Vacuolar Proteins CvpA-F, have been described to modulate the membrane identity, size, and heterotypic and homotypic fusion of the CCV with other membrane compartments within the infected host cell. The *Coxiella* effector CvpG is recruited to the CCV membrane when ectopically expressed as a fusion with eGFP, mCherry, or mClover3. CvpG-GFP preferentially localizes to perinuclear, Golgi-associated Transferrin-receptor and WGA-positive vesicles in uninfected HeLa cells and remains on CD71-marked tubules distinct from the Golgi complex after Brefeldin A treatment. The amount of CvpG protein within the host cell appears to impact homotypic fusion of the CCV - abundant expression of CpvG-GFP in *Coxiella* infected cells results in smaller, more abundant CCVs. On the other hand, when native *Coxiella* expression of CvpG is disrupted by a transposon, CCVs are enlarged and recruit more LAMP1 and Rab9a proteins. Compared to CCVs formed by wild-type *Coxiella*, CvpG::tn CCVs have lower pH as measured by pHrodo-dextran and GFP-RFP-LC3 fluorescence. Finally, CvpG-disrupted strains have decreased virulence in a *Galleria* infection model. Thus, CvpG is a the seventh described Coxiella Vacuolar Protein (Cvp) and modulates both homotypic and heterotypic vesicle fusion with the Coxiella Containing Vacuole.

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The IMPACT of Coxiella: Learning from a pathogen during a pandemic

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Coxiella burnetii secretes >150 effector proteins into the eukaryotic host cell, interacting with all major membrane-bound organelles to potentiate a successful infection. We defined a repertoire of 168 putative effectors in the RSA 493 genome and found both concordant and discordant functional characterization in 13 years of published studies. We wondered – could students learn molecular biology, biochemistry and cell biology while also validating the predictions and observations of researchers? Could Intracellular Microbial Pathogens Advance Cell-biology Teaching to IMPACT student learning? As a remote and hybrid research group, we compared predicted and observed effector secretion, function and localization. Two online platforms - S4TE and Bastion – correctly predicted 96% of the experimentally validated *dot/icm* secreted effectors. 27% of the putative effectors were not analyzed for secretion, and 54% of the effector repertoire lacks any functional characterization. To form hypotheses about uncharacterized effector function, PredictProtein was used to determine the expected features and eukaryotic cell localization of all 168 proteins. Comparing to published results, we found 33 concordant predictions, 21 discordant, and 32 effectors with predicted localization to a eukaryotic organelle but no experimental characterization. Simultaneously, a modular half-semester tissue culture and microscopy lab course appropriate for undergraduates was developed, including transfection and visualization of eukaryotic organelles. In our next phase, we will clone and ectopically express monomeric fluorescent mClover3-effector fusions in HeLa cells, beginning with 25 proteins we predict will interact with membrane-bound organelles. AT-rich *Coxiella* genes are poorly expressed in human cells, and codon-optimized synthetic genes were necessary to improve protein expression. Cloning, sequencing and verification of fusion proteins will serve as additional modules in a full-semester course. Eventually, IMPACT-CURE students will build, express, image, and validate a library of all *Coxiella* effector proteins. Collaborations involving both research and teaching are welcomed for this project.

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OMP-1 and VirB2 Immunization of Dogs Minimizes Tick-Transmission of *Ehrlichia*

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Ehrlichia chaffeensis is an obligatory intracellular bacterium that causes human monocytic ehrlichiosis, an emerging disease transmitted by the Lone Star tick, *Amblyomma americanum.* Among 22 paralogs of *E. chaffeensis* porin P28s/OMP-1s, OMP-1B is the only one expressed by *E. chaffeensis* in *A. americanum* ticks. Type IV secretion system (T4SS) is essential for *E. chaffeensis* infection of host cells. In this study we investigated potentials of OMP-1B and VirB2-4, a pilus protein of T4SS, as vaccine candidates against *Ehrlichia*. Recombinant OMP-1B (rOMP-1B) and recombinant VirB2-4 (rVirB2-4) immunization protected mice from *E. chaffeensis* infection as effective as Entry-triggering protein of *Ehrlichia* immunization (1). Dogs vaccinated with a nanoparticle vaccine composed of rOMP-1B or rVirB2-4 with immunostimulating complex developed high antibody titers against the respective antigen. All dogs including sham-vaccinated with the complex alone were challenged with *A. americanum* adult ticks infected as nymphs with *E. chaffeensis*. At day 3 post tick attachment (pta), all four sham-vaccinated dogs were infected, whereas *E. chaffeensis* was undetectable in two of three rOMP-1-immunized dogs. *E. chaffeensis* was not detected in all three rVirB2-4-immunized dogs at day 3 pta until termination of the experiment. *E. chaffeensis* in transmission-fed ticks removed from OMP-1B-immunized dogs showed significantly lower bacterial load compared to ticks removed from sham-immunized dogs, suggesting in-tick neutralization. Peripheral blood leukocytes from rVirB2-4-vaccinated dogs secreted significantly elevated interferon-γ before and soon after tick attachment by ELISpot assay and RT-qPCR, suggesting interferon-γ-mediated *Ehrlichia* inhibition. The results demonstrate these *Ehrlichia* surface-exposed outer membrane proteins are potential ehrlichial tick-transmission blocking vaccine candidates.

(1) Budachetri, K, O. Teymournejad, M. Lin, Q. Yan, M. Mestres-Villanueva, G. N. Brock, and Y. Rikihisa. (2020) An Entry-triggering Protein of *Ehrlichia* is a new vaccine candidate against tick-borne human monocytic ehrlichiosis. mBio. 11: e00895-20.

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The Interplay between flea-borne Rickettsiae

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The objective of the current investigation was to determine the following: 1) the presence, distribution and prevalence of flea-borne rickettsiae (*Rickettsia typhi, Rickettsia felis, Rickettsia asembonensis* and *Candidatus* Rickettsia senegalensis) in various regions around the world; 2) the effect of one (or a combination of each flea-borne rickettsial species) has on the presence, prevalence and distribution of the other flea-borne rickettsiae; 3) how that interaction, if there is any, might relate to the presence, prevalence and/or distribution of the agents within their vertebrate hosts; and 4) the impact of the presence of the various flea-borne rickettsiae on the presence of flea-borne rickettsioses. The investigation was pursued by the assessment of over 200 peer-reviewed articles for 7 regions, including 6 continents and the islands of the Pacific, Indian and Atlantic Oceans. All four flea-borne rickettsiae were present in each of the continents of Africa, Asia, Europe, North America, and South America as well as among various islands. In Australia and New Zealand both *R. typhi* and *R. felis* exist*,* there is one report of *Ca*. R. senegalensis in Australia and no reports of *R. asembonensis*. Distribution of the flea-borne rickettsiae in the reported areas of investigations do not necessarily represent entire locations due to the limited data availability. Additionally, it was noted that variability exists in the prevalence of the agents from location to location. However, the effect on the presence, distribution and prevalence of one flea-borne rickettsia on the others does not appear to be relevant, as the agents could all be found within a location. Lastly, since the flea-borne agents have little affect on the presence of the others, it has been observed that murine typhus and flea-borne spotted fever can occur in the same location.

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Endothelial mTOR activation with *R. rickettsii* strains of varying virulence: possible roles in the modulation of autophagy, inflammation, and rickettsial replication

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Vascular inflammation and dysfunction represent salient features of rickettsial pathogenesis, and details of fundamentally important pathogen interactions with host endothelial cells (EC) as the primary targets of infection remain poorly appreciated. Mechanistic target of rapamycin (mTOR), a serine/threonine protein kinase of the phosphatidylinositol kinase-related kinase family, is implicated in the determination of innate immune responses to intracellular pathogens via transcriptional regulation. We have recently shown that rickettsial infection of ECs increases mTOR phosphorylation. Here, we infected endothelial cells with *R. rickettsii* strains of varying virulence to identify differences in the kinetics and/or intensity of mTOR to establish a correlation between the host cell's mTOR response and rickettsial virulence. Endothelial mTOR activation with virulent strains of *R. rickettsii* (HLP and Sheila Smith) was higher than the avirulent strain (Iowa). Similarly, there was increased lipidation of LC3-II with virulent strains (HLP and SS) as compared to avirulent Iowa strain of *R. rickettsii*. We next investigated the effects of mTOR inhibitors, Rapamycin and Torin2 on rickettsial replication in EC. Intriguingly, both inhibitors enhanced intracellular growth and replication as evidenced by about 2.5-fold increase in the copy numbers at 48h post-infection. To further study the role of mTOR in infection-induced inflammation, we assessed the effect of rapamycin on STAT3 activation. Rapamycin significantly attenuated the activation of STAT3 as measured by its nuclear translocation and binding to a consensus oligonucleotide probe in a gel-shift assay. Also, mTOR inhibition resulted in increased expression of pro-inflammatory cytokines, Interleukin (IL)-1α and IL-6, as measured by qPCR. These findings thus yield first evidence of differential activation of mTOR during cellular infection with rickettsial strains of varying virulence and suggest that early induction of autophagy in response to intracellular infection might be regulated by this important pathway known to function as a central integrator of cellular immunity and inflammation.

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Arthropod vectors: More than mobile syringes

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The arthropod-borne pathogen life cycle requires that microbes sense and adapt to disparate environmental cues. The environmental milieu between vertebrate hosts and arthropod vectors differs significantly with disparities in body temperature, physiological architecture, immunological potential, and nutrient availability. Much of what is known about vector-borne pathogens is centered around interactions with mammalian hosts. Comparatively less is understood about arthropod interactions. This is a significant knowledge gap as the vector plays a crucial role in not only harboring pathogens, but also priming them for transmission. Based on historical findings and recent developments, vector-microbe interactions can be broadly described in three categories: barriers to colonization, microbial manipulation, and saliva-transmission dynamics. The work in my lab has focused on the first of these categories by investigating how the arthropod responds to pathogen colonization and defining whatforces shape these interactions. From this work, we have found that cellular stressforces shape these interactions. From this work, we have found that cellular stress reveal that divergent tick-transmitted microbes trigger the unfolded protein response, which activates the immune deficiency pathway. This is a departure from current thinking in arthropod immunity. In this scenario, stress caused by infection triggers the immunocircuitry, rather than microbial-derived PAMPs (pathogen-associated molecular patterns). Cellular stress responses are not necessarily specific to different types of stressors and instead respond by monitoring macromolecular damage to the cell. This more generalized signal widens the infection-sensing scope of possibility and, from this perspective, reduces the requirement for an array of specific immune receptors. In the second part of this talk, I will discuss recent findings pertaining to the second two categories of microbe-arthropod interactions, highlighting work that describes 1) microbial manipulation of the vector, and 2) how arthropod saliva promotes rickettsial transmission and disease severity.

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The retropepsin-type protease APRc as a novel Ig-binding protein and moonlighting immune evasion factor of *Rickettsia*

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Rickettsiae are obligate intracellular Gram-negative bacteria transmitted by arthropod vectors. Despite their reduced genomes, the function(s) of the majority of rickettsial proteins remains to be uncovered. APRc is a highly conserved retropepsin-type protease, suggested to act as a modulator of other rickettsial surface proteins with a role in adhesion/invasion. However, APRc's function(s) in bacterial pathogenesis and virulence remains unknown. This study demonstrates that APRc targets host serum components, combining non-immune immunoglobulin (Ig)-binding activity with resistance to complement-mediated killing. We confirmed non-immune human IgG binding in extracts of different rickettsial species and intact bacteria. Our results revealed that the soluble domain of APRc is capable of binding to human (h), mouse, and rabbit IgG and different classes of human Ig (IgG, IgM, and IgA) in a concentration-dependent manner. APRc-hIgG interaction was confirmed with total hIgG and normal human serum. APRc-hIgG displayed a binding affinity in the micromolar range. We provided evidence of interaction preferentially through the Fab region and confirmed that binding is independent of catalytic activity. Mapping the APRc region responsible for binding revealed the segment between amino acids 157-166 as one of the interacting regions. Furthermore, we demonstrated that expression of the full-length protease in E. coli is sufficient to promote resistance to complement-mediated killing and that interaction with IgG contributes to serum resistance. Moreover, our results point towards cleavage of other serum components by APRc. Two of these putative substrates are currently being characterized and investigated for their role in enhancing immune evasion. Our findings position APRc as a novel Ig-binding protein and a novel moonlighting immune evasion factor of *Rickettsia*, contributing to the arsenal of virulence factors utilized by these intracellular pathogens to aid in host colonization.

COMPETE 2020 - Operational Programme for Competitiveness and Internationalisation & Portuguese national funds via FCT, under project[s] POCI-01-0145-FEDER-029592/PTDC/SAU-INF/29592/2017& UIDB/04539/2020/UIDP/04539/2020 (CIBB).

Assessment of the pathogenicity of purportedly nonpathogenic *Rickettsia* via tick bite from naturally infected vectors

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While case fatality rates in the United States for Spotted Fever Rickettsiosis (SFR) have remained low since the advent of effective antibiotics, the number of reported cases has increased over the last 20 years. This suggests that less virulent species may contribute to the recent rise of cases. Serological evidence supports this hypothesis; infection with purportedly non-pathogenic *Rickettsia* species can cause seroconversion that cross-reacts when analyzed using standard diagnostic tests, such as indirect immunofluorescence assays. Additionally, there is evidence that these purportedly non-pathogenic *Rickettsia* (such as *Rickettsia amblyommatis*) cause mild illness in model animals and humans. However, much of this animal work involved needle inoculation, which may cause a different clinical course than tick bite inoculation. With this in mind, we fed field-collected adults of common human-biting tick species on guinea pigs. Infectious agents were identified via PCR both in engorged ticks and animal tissues. Clinical signs were recorded for 2-to-3-weeks following tick attachment. Comparisons between *Rickettsia* infected and *Rickettsia* uninfected tick feedings were made for *Dermacentor variabilis* infected with *R. montanensis* and *R. bellii* and *Amblyomma americanum* infected with *R. amblyommatis*. There was no statistical significance for clinical signs between guinea pigs exposed to infected and uninfected ticks. However, clinical signs were noted in both treatment groups, suggesting that possibly tick feeding affects vertebrates or other agents present produced signs of infection. Clinical profiles were similar when compared to guinea pig studies using intraperitoneal inoculation of these same rickettsial species. However, an increase was observed in dissemination and subsequent molecular detection of *Rickettsia* in the tick-exposed animals' skin, suggesting that tick-borne transmission changes the infection profile. Salivary proteins active during tick feeding likely affect the clinical profile, as previously reported in other arthropod and vector-borne disease models. Assessment of infection associated with other potentially disease-causing agents is also warranted.

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Coxiella burnetii manipulates the type I interferon pathway in a T4BSS dependent manner

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Coxeilla burnetti is an obligate intracellular pathogen that is the etiological agent of Q fever.Inside the host cell, it resides and replicates in a large acidic vacuole, designated as the Coxiella-containing vacuole (CCV). Coxiella subverts the immune system, manipulates the host, and maintains the CCV through various effectors secreted by the Dot/ICM type IVb secretion system. A handful of effectors have been shown to manipulate host immune pathways, but none have been directly connected to the type I interferon (IFN) pathway, which may impact disease progression. Here we show that macrophages infected with a dotA::Tn Coxiella mutant produce significantly higher levels of ifnb and interferon-stimulated genes compared to macrophages infected with wild-type NMII Coxiella. This strongly suggests the type IVb secretion system and/or its associated effectors inhibit macrophages' ability to produce type I IFNs in response to Coxiella infection. In further exploring the mechanism for this inhibition, we identified three specific transposon mutants with defective effector expression that were also unable to block expression of IFNb and interferon-stimulated genes. Importantly, in IFNAR-/- macrophages, type I IFNs are no longer expressed after Coxiella infection, suggesting this finding is specific to the type I ifn pathway and not due to another adjacent immune pathway. Collectively our results show Coxiella effectors manipulate the type I IFN response during infection of macrophages and reveal a novel strategy it uses to manipulation of host biology.

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Pathogenic *Rickettsia* species manipulate host immune defense pathways to facilitate intracytosolic replication within the host.

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Rickettsia species are strict obligate intracellular bacteria and exhibit a range of virulence from harmless endosymbionts of arthropods to the etiologic agents of severe disease, including humans. One critical feature of these stealthy group of pathogens is their ability to manipulate hostile cytosolic signaling to their benefits. In fact, preceding data from our laboratory and others have shown that pathogenic *Rickettsia* species utilize secreted effectors to facilitate colonization by manipulating ER structures or by modulating intracellular trafficking to manipulate host defense pathways. However, the mechanisms of host innate immune defense evasion by pathogenic *Rickettsia* species remains to be elucidated. To address this question in a more comprehensive manner, we tested the hypothesis that pathogenic, but not non-pathogenic, *Rickettsia* spp. evade immune responses in host defense cells, like macrophages, to replicate and disseminate. For this purpose, we evaluated the cytosolic host defense responses between pathogenic *R. rickettsii* (Sheila Smith) and *R. typhi* (Wilmington) and non-pathogenic (*R. montanensis*) strains *in vivo and in vitro*. We showed that disease severity in wild-type (WT) C57BL/6J mice infected with *R. typhi* and *R. rickettsii*, but not with non-pathogenic *R. montanensis*, correlated with levels of bacterial burden as detected in the spleens, as well as the serum concentrations of pro-inflammatory cytokines, including IL-1α and to a lesser extent IL-1β. Antibody-mediated neutralization experiments identified IL-1α as a key mediator to control mortality rates and bacterial burdens of rickettsiae-infected mice. As macrophages are a primary source of both IL-1α and IL-1β cytokines, we determined the mechanism of the anti-rickettsial activities using bone-marrow-derived macrophages. We found that pathogenic *R. typhi* and *R. rickettsii*, but not non-pathogenic *R. montanensis*, induced autophagy, and avoided autophagolysosomal destruction, while simultaneously eluded pro-IL-1α induction and benefited from a reduced IL-1α secretion, via a Caspase-11-Gsdmd-dependent mechanism, to facilitate intracytosolic replication. In sum, we identified a previously unappreciated pathway by which pathogenic, but not non-pathogenic, *Rickettsia* preferentially target the non-canonical inflammasome-IL-1α signaling axis in macrophages, possibly via an autophagy-dependent mechanism, to support their replication and dissemination within the host.

A murine model of waning scrub typhus cross-protection between heterologous strains of *Orientia tsutsugamushi*

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Scrub typhus, a life-threatening mite-borne zoonosis, is an underreported febrile disease with no licensed vaccine. The etiologic agent, *Orientia tsutsugamushi*, is an obligately intracellular bacterium with numerous strains that have antigenic heterogeneity. Advancement towards a protective vaccine against scrub typhus has been encumbered by the strain diversity and a gap in knowledge regarding the mechanism of waning immune protection to natural infection. Short-lived protection against infections with the homologous strain in humans has been described with a reported mean antibody reversion rate of less than one year. Protection from a heterologous strain is inadequate with rickettsemia as early as one month and development of scrub typhus disease after one year upon challenge. Geographical overlap of numerous strains of *O. tsutsugamushi* warrant the need for a vaccine that would afford long-lived cross-protection among strains. To enhance the understanding of this transient immunity, animal models demonstrating cross-protection and the deterioration of this cross-protection are necessary. We sought to utilize our sublethal intradermal infection model of scrub typhus followed by challenge with an ordinarily lethal hematogenously disseminated infection to establish a model to build upon our current understanding of this ineffective protection. An initial sublethal infection with *O. tsutsugamushi* Gilliam strain afforded robust protection from illness upon rechallenge with 5 LD_{50} *O. tsutsugamushi* Karp strain after one month accompanied by marked splenomegaly, significantly increased circulating white blood cells, and abundant mononuclear cellular infiltrates in target organs such as lung, liver, and kidney. However, signs of illness as well as weight loss were observed three-months after the initial Gilliam strain infection and were more severe at nine- and 14-months. Animals were protected from death until 14-months after the initial Gilliam strain infection. The protection was not sterile as Karp strain bacteremia was observed at all time-points.

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Expression of *Orientia tsutsugamushi* p56 antigen in recombinant non-replicative adenovirus virus vector for evaluation of protection against scrub typhus infection

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Orientia tsutsugamushi is the etiologic agent of scrub typhus, which is the most prevalent rickettsiosis worldwide. *Orientia* is maintained transovarially by its reservoir hosts, trombiculid mites, which emerge as larvae from the soil seeking a meal. Humans develop an eschar at the site of inoculation, and subsequently the bacteria disseminate hematogenously throughout the body with infection of endothelial cells and macrophages. P56 is a major immunodominant, antigenically diverse protein containing 4 hypervariable regions. It plays a primary role in adhesion and entry of the obligately intracellular bacteria. Additionally, it has been found to stimulate strong protective immunity against lethal challenge with the *O. tsutsugamushi* homologous strain. Previous publications have provided evidence that neutralizing epitopes are located in hypervariable regions 2 and possibly 1 and/or 3. A replication-deficient recombinant human adenovirus 5 vector expressing P56 of *O. tsutsugamushi* Karp strain was prepared and the entire ORF of P56, a 1599 base pair fragment, was directionally cloned into pAdenoX-ZsGreen-1, an adenoviral expression vector designed to constitutively express a gene of interest and ZsGreen 1 (green fluorescent protein from *Zoanthus sp.*) in mammalian cells. Recombinant adenoviral plasmid DNA was purified after PCR confirmation that the encapsidated adenoviral genome contained a functional copy of the sequence of interest. Transfection and amplification of the recombinant adenoviral particles into human embryonic kidney cells (Adeno-X-293) was performed. High titer stocks were produced with several cycles of transfection, and protein lysates were screened by western blot analysis. A protein band of the expected size was detected in P56 lysate and not in non-recombinant lysate when reacted with rabbit polyclonal sera against *O. tsutsugamushi* Karp strain. Our aim is to develop a vaccine directed against neutralizing epitopes on P56 to provide protection against lethal challenge with the homologous strain of *O. tsutsugamushi*.

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Type VIB Secretion System Protein, icmE Contribute to *Coxiella burnetii* virulence, intracellular replication, and activation of inflammasome mediators during infection

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Coxiella burnetii is an obligate intracellular Gram-negative bacterium that causes Q-fever, a life-threatening zoonotic disease. *C. burnetii* replicates within an acidified parasitophorous vacuole derived from the host lysosome. The ability of *C. burnetii* to replicate and make successful intracellular life in host cytosol is vastly dependent on the Dot/Icm type 4B secretion system (Dot/Icm T4SS). Although few T4SS proteins have been shown to be important for *C. burnetii* virulence and intracellular replication, the role of icmE protein in host-*C. burnetii* interaction remains unknown. In this study, we constructed a *C. burnetii* Nine Mile phase II (NMII) mutant library and identified 146 transposon mutants with a single transposon insertion. The transposon mutagenesis screen revealed that the disruption of the icmE gene resulted in attenuation of *C. burnetii* NMII virulence in SCID mice. Magpix Luminex cytokine assay indicated that the decreased secretion of inflammatory cytokines including interleukin-1β, IFN-γ, TNF-α, and IL-12p70 in serum from Tn::icmE mutant-infected SCID compared to mice infected with wild-type (WT) *C. burnetii* NMII. The replication of the Tn::icmE mutant was found to be inhibited *in vitro* using mouse bone marrow-derived macrophages (BMDMs) and human macrophage-like cell lines (THP-1). The immunoblotting results showed that the Tn::icmE mutant failed to activate inflammasome components such as IL-1β, caspase 1, and gasdermin-D in THP-1 macrophage cells. Together, these results strongly suggest that *Coxiella* icmE protein may play an important role for *C. burnetii* virulence, intracellular replication, and activation of inflammasome mediators during infection.

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Sticking together – *Ixodes pacificus* and symbiotic *Rickettsia monacensis* strain Humboldt

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Nutritive symbiosis between bacteria and ticks is observed across a range of microbes and hosts, however, little characterization on the molecular components responsible for these symbiosis has been done. Previous studies in our lab demonstrated *Rickettsia monacensis* str. Humboldt has a genetic capacity of synthesizing folate de novo folate biosynthesis pathway that is composed of *folA*, *folC*, *folE*, *folKP*, and *ptpS* genes. In this study expression of folate genes of str. Humboldt, within folate gene mutant *E. coli* K-12 str. BW25113, was used to functionally characterize folate genes of str. Humboldt in vivo. Folate genes of str. Humboldt were subcloned into TransBac vector and transformed into *folA*, *folC*, *folE* or *folK* knockout mutant *E. coli* K-12 str. BW25113. Each mutant containing a folate gene subclone and pFE604 clone of knocked-out folate gene was cured of pFE604. Curing of *E. coli folA* and *folC* mutant was successful using acridine orange and 43.5°C but curing *folK* mutant was only achieved after supplementing curing media with thymidine, a folate end-product. Preliminary plasmid curing assay showed curing efficiency of *E. coli folA* mutant at 100%, whereas curing efficiencies for other *E. coli* folate mutants was 0% to 6%. Cured candidates were confirmed by colony PCR assay and/or real-time quantitative PCR assay. Functional complementation was assessed by growth phenotype on minimal media with and without IPTG between *folA* of str. Humboldt and *E. coli folA* as well as *folC* of str. Humboldt and *E. coli folC* gene pairs. Large and homogenous wild-type colony growth was observed for both assayed gene pairs on minimal media with IPTG, and lack of growth or pin-point growth without IPTG. This study provides evidence substantiating the in vivo functionality of folate genes of *R. monacensis* str. Humboldt in producing functional gene products for folate biosynthesis.

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Ehrlichia chaffeensis TRP120-mediated ubiquitination and degradation of transcriptional corepressor TLE4

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Ehrlichia chaffeensis modulates numerous host cell processes, including gene transcription to promote infection of the mononuclear phagocyte. Modulation of these host cell processes is directed through *E. chaffeensis* effectors, including TRP120. We previously reported that TRP120 moonlights as a HECT E3 Ub ligase that ubiquitinates host cell transcription and fate regulators (PCGF5, FBW7 and ENO-1) which enhances infection and also revealed that *E.chaffeensis* activates canonical Wnt signaling in monocytes to promote bacterial uptake and intracellular survival. Further studies have shown that siRNA knockdown of transducin-like enhancer of split 4 (TLE4) coincided with increased E. chaffeensis infection. However, how the TLE4 is inactivated or displaced from T-cell factor/lymphoid enhancer factor (TCF/Lef) transcription factors binding complexes and affect Wnt-specific transcription program is poorly understood. In this study, we examined relationship between TRP120 and TLE4 that functions as a transcriptional repressor. TLE4 degradation was observed during infection and was inhibited by the proteasomal inhibitor bortezomib. A direct role of TRP120 Ub ligase activity in TLE4 degradation was demonstrated and confirmed by ectopic expression of TRP120 HECT Ub ligase catalytic site mutant. Immunofluorescence microscopy and coimmunoprecipitation demonstrated TRP120 interacts TLE4, and ubiquitination of TLE4 by TRP120 was detected *in vivo* and *in vitro*. This investigation further expands the repertoire of TRP120 substrates and extends the potential role of TRP120 Ub ligase in infection to include how evolutionarily conserved signaling pathways are hijacked by obligately intracellular pathogens.

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Orientia tsutsugamushi host-pathogen interactions and new tools for discovery

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Scrub typhus is an emerging and potentially deadly infection that has been dubbed by the World Health Organization as one of the most underreported diseases requiring hospitalization. An etiologic agent is *Orientia tsutsugamushi*, an obligate intracellular bacterium that is transmitted by mites and colonizes leukocytes and endothelial cells in mammalian hosts. Its evolution as an endosymbiont of arthropods and mammals engendered its survivability in diverse host cells and success as a pathogen in accidental human hosts. *O. tsutsugamushi* modulates immune responses and other cellular processes using mechanisms that are poorly understood. The ankyrin repeat is one of the most common protein-protein interaction motifs and is conserved across the Tree of Life. *O. tsutsugamushi* encodes one of the largest cadres of ankyrin repeat-containing effectors (Anks) among bacteria, a striking revelation given the dogma that "obligates" jettisoned non-essential genes during their reductive evolution. For the past decade, our lab has investigated the overarching hypothesis that *O. tsutsugamushi* Anks mediate host-pathogen interactions that enable the microbe to survive intracellularly, counter immunity, and cause disease. This talk will review our progress on this front, focusing on Anks that modulate innate and adaptive immunity, the contributions of these effectors' other eukaryotic-like domains, and approaches that can be applied to interrogate Ank function during infection while circumventing *O. tsutsugamushi* genetic intractability. We will also discuss how our and others' findings imply that differential carriage or expression of Anks by *O. tsutsugamushi* strains potentially influence pathogenicity. Finally, we will present our progress on developing tools for genetically manipulating this organism.

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Moonlighting on Steroids: *Ehrlichia* TRP Effectors, SLiMs and PTMs

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The first *Ehrlichia chaffeensis* tandem repeat protein (TRP120) was molecularly identified and cloned in 1997 by rickettiologist, Xue-jie Yu. TRP120 was identified as an immunoreactive protein and studies thereafter demonstrated expression of the surface of dense-cored ehrlichiae and secretion into the host cell. In the 2000's, TRP120 nuclear localization and nucleomodulin activity was demonstrated, including host DNA binding and targeting of genes associated with transcriptional regulation, signal transduction and apoptosis. Subsequently, TRP120 was also shown to have E3 ubiquitin ligase activity and several host substrates were identified. Most recently, TRP120 has been identified as a eukaryotic ligand mimetic that activates multiple host cell signaling pathways via short linear motifs (SLiMs). The multiple functions and multitude of host interactions suggested in early studies of TRP120 can now be rationally explained by disordered protein structure, post translational modifications (PTMs), and SLiMs. Twenty-five years later, TRP120 is a well-defined moonlighting protein, but our understanding of TRP120 during infection is not complete. Potential new host interactions that are illuminated by the multiple layers of complexity including protein plasticity, PTMs, and large numbers of SLiMs, both defined and undefined, suggest that currently known TRP120 functions are simply the beginning of a much more expansive and complex role in pathobiology. Using TRP120 as a model to understand the nature of disordered proteins, PTMs, and SLiMs in pathogen host interactions has tremendous potential for revealing molecular infection strategies that can impact rickettsiology and beyond.

EHRLICHIA SLIM LIGAND MIMETIC ACTIVATES NOTCH SIGNALING IN HUMAN MONOCYTES

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Ehrlichia chaffeensis evades innate host defenses by reprogramming the mononuclear phagocyte through mechanisms that involve exploitation of multiple evolutionarily conserved cellular signaling pathways including Notch. This immune evasion strategy is directed in part by tandem repeat protein (TRP) effectors. Specifically, the TRP120 effector activates and regulates Notch signaling through interactions with the Notch receptor and the negative regulator, F-Box and WD repeat domain-containing 7 (FBW7). However, the specific molecular interactions and motifs required for *E. chaffeensis* TRP120-Notch receptor interaction leading to activation have not been defined. To investigate the molecular basis of TRP120 Notch activation, we compared TRP120 with known endogenous canonical/non-canonical Notch ligands and identified a short region of sequence homology within the tandem repeat (TR) domain. Using Informational Spectrum Method (ISM) analysis, TRP120 was predicted to share biological function with Notch ligands, and cross spectral analysis identified a function-associated sequence in the TR domain. To investigate TRP120 and Notch receptor interactions, colocalization between ectopically expressed full length TRP120 and the Notch-1 receptor ligand binding region (LBR; EGF repeats 1-15) was observed. Moreover, direct interactions between recombinant full length TRP120, the TRP120 TR domain containing the putative Notch ligand sequence, and the Notch receptor LBR were demonstrated using pull-down, surface plasmon resonance and protein-coated fluorosphere approaches. In order to molecularly define the TRP120 Notch activation motif, overlapping and reductive peptide mapping was used to identify an 11-amino acid short linear motif (SLiM) located within the TRP120 TR that was fully capable of activating Notch signaling and downstream gene expression in THP-1 cells and primary human monocytes. Peptide mutants of the Notch SLiM or anti-Notch SLiM antibody reduced or eliminated Notch activation and NICD nuclear translocation. This investigation reveals a novel molecularly defined pathogen encoded Notch SLiM mimetic that activates Notch signaling consistent with endogenous ligands.

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Probing rickettsia pathogenesis using advanced biochemical and genetic tools

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Spotted Fever Group *Rickettsia* species evolved dynamic strategies to invade host cells, escape into the cytosol, and spread from cell to cell. This complex intracellular life cycle likely relies on an arsenal of secreted effectors that reprogram host cell processes. Unfortunately, the identity and host targets of these secreted effectors have remained largely unknown due to challenges in growing and genetically manipulating these pathogens in the lab. To overcome this issue, my lab is using a library of transposon mutants to elucidate the function of predicted or known secreted effectors and the Type IV Secretion System. As a complement to this work, we have established a modern proteomic approach to identify new *R. parkeri* secreted effectors. Our screen has revealed several novel effectors that each localize to distinct subcellular compartments, suggesting they target different host factors to support infection. We are also developing genetic tools to enable functional dissection of these effectors. In the end, our work has improved our fundamental understanding of rickettsia-host interactions, and may reveal therapeutic targets to prevent or treat rickettsiosis.

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Coxiella burnetii polysaccharide vaccine protects guinea pigs from coxiellosis

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Coxiella burnetii which causes the human disease Q fever, is an important world-wide zoonosis. Infected, parturient vertebrate animals, especially domesticated ruminants, contaminate the environment as they give birth. Q vax is an effective human vaccine used only in Australia but in no other country due to difficulties associated with its use, including pre-screen testing and the risk of adverse reactions. Hence the need for a new human vaccine that can be licenced for worldwide use. An experimental vaccine, prepared from the cell-wall polysaccharide (O-antigen) of virulent (phase 1) *C. burnetii*, strain Nine Mile, conjugated to a protein (tetanus toxoid), was used (with and without alum adjuvant) to vaccinate guinea pigs prior to intranasal challenge with *C. burnetii*.The vaccine protected guinea pigs from fever and weight loss when challenged with an infectious dose of *C. burnetii*. The spleen, liver and kidney of vaccinated guinea pigs contained significantly fewer *C. burnetii* DNA than those of unvaccinated control animals. A conjugate, polysaccharide vaccine from the cell wall of phase 1 *C. burnetii* protected guinea pigs from coxiellosis and may prove to be useful in humans.

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Cat flea coinfection with *Rickettsia felis* and *Rickettsia typhi*

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Flea-borne rickettsioses collectively referred to as the term for the etiological agents, *R. felis, R. typhi*, and RFLOs, has become a major public health concern in southern Texas and California, as well as Hawaii. Due to a shared transmitting vector (the cat flea) and clinical signs, discriminating between the agents has proven difficult. Furthermore, a paradigm shift in transmission cycles for *R. typhi* induced by changing social and environmental factors after WWII, along with increasing cases of flea-borne rickettsioses in endemic areas, has brought into question the role of other closely related flea-borne rickettsiae, such as *R. felis*. Due to the promiscuous nature of cat fleas, they often partake in multiple bloodmeals, increasing the likelihood of acquiring several pathogens throughout their lifespan. The effects of vector coinfections can result in antagonistic or synergistic relationships between organisms, ultimately altering potential human exposure and disease pathology. However, the impact of bacterial interactions among fleas remains poorly understood. Therefore, an *in vitro* and *in vivo* arthropod system was utilized in the current study to assess coinfection interactions. Coinfection within an arthropod cell line revealed the ability of *R. felis* and *R. typhi* to cohabitate in the same cell, but distinct growth kinetics led to reduced *R. felis* growth over time. Conversely, sequential flea coinfections revealed the vector could acquire both *Rickettsia* spp. and sustain coinfection for up to 2 weeks. Rickettsial loads in coinfected fleas and feces were altered during coinfection, suggesting *R. felis* and *R. typhi* interactions may enhance the transmission potential of either agent. Thus, the study presented here provides functional comprehensive analyses of *in vitro* and flea coinfection bioassays to model transmission events that will guide the understanding of the current epidemiology of flea-borne rickettsial infections.

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The intracellular lifecycle of *Orientia tsutsugamushi*

Jeanne Salje, Rutgers

Orientia tsutsugamushi (Ot) is an obligate intracellular alpha-proteobacterium in the order Rickettsiales, family Rickettsiaceae that causes the life-threatening vector-borne human disease scrub typhus. Ot differs from other Rickettsiaceae in using microtubules to move around cells, replicating in a tight microcolony in a perinuclear region, and exiting infected cells using a budding mechanism. The genome of Ot is also unusual, being nearly double the size of most other Rickettsiales due to a highly proliferated mobile genetic element. In this overview talk I will describe the current state of the field in terms of the genomics and cell biology of Ot. I will then discuss recent results from our group on the intracellular infection cycle of Ot, as well as the mechanism of microtubule-driven motility.

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Potential Vaccine Targets in VirB10 of the *Anaplasmataceae*

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Tick-borne pathogens in the family *Anaplasmataceae* cause globally significant infections of domestic animals and humans, including the emerging U.S. diseases of anaplasmosis and ehrlichiosis and heartwater disease of livestock in Africa. Effective vaccines against the *Anaplasmataceae* are not available. Molecular investigations have revealed that immunodominant surface proteins of these organisms are often highly variable, which has stimulated the search for suitable conserved vaccine targets. One such target is the Type IV secretion system, a nanomachine spanning the two membranes of Gram-negative bacteria, responsible for translocation of effectors that enable colonization and survival of bacteria in the host cell. In *Escherichia coli* the crystallographic structure of the complete outer membrane complex of Type IV was determined and established VirB10 as comprising the outer membrane channel. Importantly, a two-helix C-terminal bundle of VirB10 was shown to be exposed extracellularly, demonstrating that it projects across the outer membrane of *E. coli*. In a recent investigation we observed that soluble recombinant VirB10 of *Anaplasma phagocytophilum*, used as a vaccine, was partially protective against challenge infection in a mouse model. Although little experimental structural information is available for VirB10 of the *Anaplasmataceae* the recent development of the AlphaFold structural prediction algorithms provides a route to compare VirB10 structures. Herein, we use AlphaFold to identify potential exposed vaccine targets within VirB10 of five different pathogens within the *Anaplasmataceae*

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An insight into the Epigenetics of *Ixodes scapularis* populations

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Tick-borne diseases are gaining relevance in the US. Interestingly, 80% of the cases are concentrated in the Northeastern and Midwestern US. Several factors play a role in an arthropod's ability to vector pathogens, which may impact pathogen distribution. Behavioral and biological differences between tick populations may be driven by epigenetics. Epigenetic variation correlates with global transcriptional changes that result in phenotypic plasticity. In bees, caste differentiation is influenced by variations in DNA methylation in response to environmental and nutritional clues. Quantitative analysis of the levels of 5mCytosine in *Ixodes scapularis* from Texas and Minnesota suggest variable levels of methylation depending on the sex and season of when ticks were collected. Further, whole genome bisulfate sequencing (WGBS) of Texas and Minnesota female and male *I. scapularis* ticks confirmed differences in the sites of DNA methylation between tick populations. Pathway Enrichment Analysis indicates that the mTOR pathway, transcriptional regulation, cell division, and chromosome organization are particularly over-represented in the hypermethylated genes in Texas when compared to Minnesota. Additionally, we have generated low coverage genomes for these tick populations to analyze the overall genetic variation between *I. scapularis* from Minnesota and Texas. By establishing the link between epigenetics/genetics and vector competence, we can develop novel approaches to reduce the capacity of ticks to vector pathogens.

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Selective fragmentation of the *trans*-Golgi apparatus by *Rickettsia rickettsii*

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Fragmentation of the Golgi apparatus is observed during a number of physiological processes including mitosis and apoptosis, but also occurs in pathological states such as neurodegenerative diseases and some infectious diseases. Virulent strains of *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever, induce selective fragmentation of the *trans*-Golgi network (TGN) soon after infection of host cells by secretion of the effector protein Rickettsial Ankyrin Repeat Protein 2 (RARP2). Remarkably, this fragmentation is pronounced for the *trans*-Golgi network but the *cis*-Golgi remains largely intact and appropriately localized. Thus *R. rickettsii* targets specifically the TGN and not the entire Golgi apparatus. Dispersal of the TGN is mediated by the secreted effector protein RARP2, a recently identified type IV secreted effector that is a member of the clan CD cysteine proteases. Site-directed mutagenesis of a predicted cysteine protease active site in RARP2 prevents TGN disruption. General protein transport to the cell surface is severely impacted in cells infected with virulent strains of *R. rickettsii*. Among the proteins showing decreased surface expression is the major histocompability complex 1 protein. Reduced surface expression of the MHC-1 due to impaired trafficking might thereby contribute to *R. rickettsii* evasion of immune surveillance. Recent studies implicate the SNARE protein syntaxin 5 in disruption of the TGN. These findings suggest a novel maniuplation of cellular organization by an obligate intracellular bacterium to determine interactions with the host cell.