Chlamydia muridarum Infection Associated Host MicroRNAs in the Murine Genital Tract and Contribution to Generation of Host Immune Response

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Problem
Chlamydia trachomatis (CT) is the leading sexually transmitted bacterial infection in humans and is associated with reproductive tract damage. However, little is known about the involvement and regulation of microRNAs (miRs) in genital CT.

Methods
We analyzed miRs in the genital tract (GT) following *C. muridarum* (murine strain of CT) challenge of wild type (WT) and $CD4^+$ T-cell deficient ($CD4^{-/-}$) C57BL/6 mice at days 6 and 12 post-challenge.

Results
At day 6, miRs significantly downregulated in the lower GT were miR-125b-5p, -16, -214, -23b, -135a, -182, -183, -30c, and -30e while -146 and -451 were significantly upregulated, profiles not exhibited at day 12 post-bacterial challenge. Significant differences in miR-125b-5p (+5.06-fold change), -135a (+4.9), -183 (+7.9), and -182 (+3.2) were observed in *C. muridarum*-infected $CD4^{-/-}$ compared to WT mice. *In silico* prediction and mass spectrometry revealed regulation of miR-135a and -182 and associated proteins, *that is*, heat-shock protein B1 and alpha-2HS-glycoprotein.

Conclusion
This study provides evidence on regulation of miRs following genital chlamydial infection suggesting a role in pathogenesis and host immunity.

Introduction

Chlamydia trachomatis (CT) is the major cause of bacterial sexually transmitted infections (STI) in humans[1] and is associated with long-term reproductive damage,[2] increased chance of acquiring HIV,[3] and development of cervical cancer.[4] In 2013, the Center for Disease Control (USA) reported 19.7 million new STI cases with approximately 63% between 15–24 years of age infected with CT.[5] To control increasing incidence rates of genital CT through effective prevention programs, it is imperative to

address gaps in our current knowledge of the underlying molecular mechanisms at the initial site of infection that contribute to anti-CT immunity.[3,6,7]

Chlamydia muridarum (*C. muridarum*), a murine pathogen used in the murine genital chlamydial infection model,[8–12] mimics several aspects of infection observed in CT-infected women including bacterial colonization/ascension, tissue histopathology, immune responses, and long-term pathological sequelae.[8] Intravaginal (*i. vag.*) inoculation of *C. muridarum* in mice results in vulvitis and vaginitis in the lower genital tract (LGT, vagina and cervix) with subsequent ascension to and infection of the upper genital tract tissues (UGT, uterine horns and oviducts).[10] Host immune responses following *C. muridarum* infection induce collateral tissue damage and sequelae that are typically non-homogeneously distributed in different segments of the reproductive tract.[9] The host immune response of the genital compartment involves migration of neutrophils and macrophages early on[11,13] with subsequent development of humoral and cell-mediated immune responses.[14–16] However, only limited information on the underlying molecular mechanisms that may modulate the anti-CT immune response after infection is currently available.

There is growing evidence that small non-coding species of regulatory RNA, that is, microRNAs (miRs) contribute to critical processes including immune cell development/function[17–19] and reproductive biology.[20–22] MicroRNAs modulate gene function post-transcriptionally by direct binding to target gene mRNA.[23,24] To this end, loss of Drosha and DICER, essential components of the RNA-induced silencing complex (RISC) responsible for generation of miRs, has been associated with alteration of lymphocyte differentiation and associated immune responses.[25,26] Expression of miR-125b in naïve $CD4^+$ T cells has been reported to regulate expression of the genes *IFNG*, *IL2RB*, *IL10RA* involved in T-cell differentiation.[27] MicroRNA-29 has been observed to control innate and adaptive immune responses against *Listeria monocytogenes* and *Mycobacterium bovis* by modulating IFN-γ mRNA.[28] Conjunctival miR expression in inflammatory trachomatous scarring following CT infection in humans has recently been characterized[29] and of the 754 miRs analyzed; 82 were found to be differentially regulated and reported to control genes involved in inflammation, fibrosis, and scarring.[29] Recently, Igietseme et al.[30] reported the role of host caspases in tissue apoptosis and associated genital chlamydial pathogenesis. Importantly, these investigators provide evidence on inactivation of DICER and involvement of miRs that regulate growth and differentiation in CT-infected mice.[30]

Given that immunologic events[10] occurring relatively early in genital chlamydial infection lead to inflammation and may influence the development of upper genital pathology,[15] additional insight into modulation of host miRs during this period may increase our understanding of this process. To address this knowledge gap, we investigated the regulation of 88 inflammation and immunopathology-associated miRs (Figure S4) in the immunologically diverse[9,14,31,32] lower 'non-sterile' (vagina and cervix) and upper 'sterile' (uterine horns and oviducts) compartments of the murine genital tract at day 6 and 12 post-*C. muridarum* challenge. Comparative profiling revealed nine miRs (miRs-125b-5p, -214, -23b, -135a, -182, -183, -30c, -30e, and -146) to be significantly regulated at day 6 post-challenge in the LGT and were assessed for probable role(s) in chlamydial ascension and host immune modulation. *In vitro* knockdown using miR-specific inhibitors was associated with significant increase in *C. muridarum* numbers in three of these nine miRs, that is, -125b-5p, -30c, and -182. Additionally, following *C. muridarum* infection, significant regulation of inflammatory molecules, that is, NF-κB (miR-125b-5p, -30c, and -182 knockdown conditions) and IRF-1 levels (miR-125b-5p knockdown conditions) were observed. STAT-1 levels were insignificantly affected upon miR-125b-5p, -30c, and -182 knockdown. Similarly, IRF-1 levels were not affected upon -30c, and -182 knockdown. Comparison of these nine miRs in wild type (WT) and $CD4^{-/-}$ animals' LGT isolated at day 6 post-*C. muridarum* challenge showed significant regulation of miR-125b-5p, -135a, -182, and -183. Further, mass spectrometric and *in silico* analysis of proteins in $CD4^{-/-}$ and WT LGT tissues suggested putative regulation of heat-shock protein B1 (HSPB1) and α2HS-glycoprotein (AHSG) by miR-135a, -182 following *C. muridarum* infection.

Methods

Ethics Statement

All experiments involving animals in this study were performed in compliance with the Animal Welfare Act, the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals, the 'Guide for

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the Care and Use of Laboratory Animals' published by the National Research Council, and guidelines set forth by the University of Texas at San Antonio Institutional Animal Care and Use Committee (IACUC) under approved protocol MU012-03/14A1.

Bacteria

Chlamydia muridarum was grown on confluent HeLa cell monolayers and purified as previously described.[33] Briefly, infected HeLa cells were lysed using a sonicator (40 V pulses for 10 s on ice; Fisher, Pittsburgh, PA, USA), and chlamydial elementary bodies (EBs) were harvested and purified on renograffin gradients. Titered aliquots of bacteria were stored at −70°C in sucrose-phosphate-glutamine buffer until used.

Mice

Four- to six-week-old female C57BL6 WT, $CD4^+$ T-cell deficient ($CD4^{-/-}$), $CD8^+$ T-cell deficient ($CD8^{-/-}$), and Interferon Gamma Receptor deficient (IFN-$\gamma R^{-/-}$) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed and bred at the University of Texas at San Antonio.

Intranasal Immunization Procedure

C57BL6 WT mice were immunized as described previously.[34] Specifically, groups of mice were anesthetized (3% isofluorane) and immunized intranasally on day 0 with 15 μg of rCPAF in 25 μL sterile PBS. On days −1, 0, and +1, 10 μg CpG containing 1% normal mouse serum was administered. Mice were boosted intranasally with the same dose on days 14 and 28. The selected rCPAF dose (15 μg/mouse) provided optimal protection against genital *C. muridarum* challenge.[33]

Intravaginal Infection

To render mice anestrous and more receptive to genital infection, animals were injected subcutaneous with 2.5 mg Depoprovera (medroxy-progesterone acetate; Pharmacia & Upjohn Co., New York, NY, USA) on day 5 before infection.[35] Mice were inoculated *i.vag.* with 5×10^4 *C. muridarum* inclusion forming units (IFU). For determination of miR dose-dependence groups of WT, mice were infected *i.vag* with 5×10^4, 5×10^5, or 5×10^6 IFU.

Bacterial Burdens

Bacterial burdens were monitored using vaginal swabs on indicated days post-*C. muridarum* challenge and subsequent plating on HeLa cell monolayers grown on culture coverslips.[36] Chlamydial inclusions were detected using an anti-*Chlamydia* genus-specific murine monoclonal primary antibody and goat anti-mouse IgG secondary antibody conjugated to Cy3 plus Hoescht nuclear stain. The average number of inclusions in five random microscopic fields was calculated for each animal for time points up to day 12 post-challenge and entire coverslips for days 15–30 post-challenge, and results expressed as average number of inclusions per animal group.[34]

RNA Isolation and miRNA PCRs

At the indicated days following infection, mice were euthanized, and the genital tract was dissected out, cut into two sections, that is, LGT and UGT, and snap-frozen until used. Total RNA was extracted from snap-frozen tissue sections using the miRNeasy RNA extraction Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. Total RNA was assessed using a Nanodrop Spectrophotometer (ThermoScientific, Asheville, NC, USA), and RNA samples (1 μg with $A_{260/280}$ and $A_{260/230}$ values of ≅ 2.0 and ≅1.8 or higher, respectively) were converted to cDNA using a RT^2 First Strand cDNA Kit according to manufacturer's instructions (Qiagen). For miRNA screening experiments, real-time quantitative PCR was performed on Murine Inflammation and Immunopathology Focused RT^2 miRNA plates (MAM 104D; Qiagen) using a RT^2 SYBR Green qPCR Mastermix per manufacturer's instructions (Qiagen) and Bio-Rad CFX96 thermocycler (Bio-Rad, Hercule, CA, USA). Individual miR PCR amplifications were performed using custom designed miScript Primer Assays (Qiagen) per manufacturer's instructions on a DNA Engine Opticon 2 continuous fluorescence detection system supported with Opticon Monitor Software v2.02 (MJ Research, Waltham, MA, USA). All miR expression analyses were normalized to housekeeping RNU6-2_1 or SNORD68 expression values and determined using $2^{(-\text{Average}\Delta\Delta C_T)}$.[37]

In vitro Inhibitor Assay

HeLa 229 cells were seeded at 2.5×10^5 cells/well in 24-well culture plates. At the time of seeding,

miRNA-specific inhibitors (miScript miRNA Inhibitor; Qiagen) were transfected at a final concentration of 20 μM per well using Attractene transfection reagent (Qiagen) as per manufacturer's specifications; transfection efficiency and knockdown were observed to be >80%. At 16 hr post-transfection, cells were infected with 0.1 MOI *C. muridarum* EBs. Infected cells were fixed 24 hr post-infection using paraformaldehyde and stained for chlamydial IFU enumeration as previously described.[34] Non-specific inhibitors (Qiagen) were used as negative control, and infectivity data calculated as the difference in *C. muridarum* IFU-infected wells and wells with miR-specific inhibitors.

In vitro CIGNAL Assay

To monitor modulation of nuclear factor, that is, NF-κB, and the signal transducer and activator of transcription, that is, STAT-1, and interferon regulatory factor, that is, IRF-1 following *C. muridarum* infection in miR knockdown conditions, dual luciferase/renilla activity for NF-κB, STAT-1, and IRF-1 was determined using respective reporter constructs in the CIGNAL Reporter Assay Kit (Qiagen), which encodes the firefly luciferase reporter gene under the control of a minimal (m)CMV promoter. Reporters for all three pathways were transfected either alone or in combination with miScript miRNA inhibitors for miR-125b-5p, -30c, and -182 in 2.5×10^5 HeLa cells. At 16 hr post-transfection, these transiently transfected cells were infected with 0.1 MOI *C. muridarum* EBs. NF-κB (6 hr post-infection), STAT-1, and IRF-1 (24 hr post-infection) activity were determined by monitoring luciferase/renilla levels using the Dual Glo Luciferase assay (Promega, Madison, WI, USA) according to manufacturer's instructions and a Synergy 2 Muti-Mode Microplate Reader (Biotek, Winooski, VT, USA).

Mass Spectrometry

For analysis of putative downselected miR targets, mass spectrometry was performed on genital tract tissue from day 6 naïve, *C. muridarum*-infected WT and $CD4^{-/-}$ mice. Total cell protein extraction was accomplished using RIPA Lysis Buffer Kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to the manufacturer's protocol. Protein concentration was determined using an Invitrogen EZQ Protein Quantitation Kit (Invitrogen, Grand Island, NY, USA). Quantitative tandem mass spectrometry-based 'microwave & magnetic (M^2)' proteomics was performed on individual specimens and pooled reference materials from two different groups of three specimens as previously described.[38] Tryptic peptides, including reference material, were terminally tagged with TMT-6plex isobaric labeling reagents (ThermoScientific, San Jose, CA). Each specimen was derivatized with one TMT reagent, that is, 127–130 while pooled reference materials were encoded with TMT reagents 126 or 131 (*C. muridarum*-infected $CD4^{-/-}$ and WT, respectively). To normalize across all specimens, TMT-encoded cell lysate was mixed with pooled reference material and subjected to triplicate analysis with capillary liquid chromatography–Fourier transform–tandem mass spectrometry (LC/FT-MS/MS).

In silico and Statistical Analyses

Bioinformatic analysis for putative binding sites in genes encoding proteins observed to be modulated by *C. muridarum* infection-related miRs was performed using a miR target predictive algorithm (www.microRNA.org, Memorial Sloan-Kettering Cancer Center, NY, USA). All experimental results are calculated on the mean ± SD of two to five independent experiments. GraphPad Prism 5 (La Jolla, CA, USA) was used to perform all tests of significance. MiR analysis was performed using RT^2 Profiler PCR Array Data Analysis version 3.5 (Qiagen). Student's *t*-test was used for comparisons between two groups and ANOVA with Tukey B *post hoc* test for three or more groups. Differences were considered statistically significant if *P* values were <0.05.

Results

Identification of Differentially Expressed Host MicroRNAs During Genital *C. muridarum* Infection

There is accumulating evidence on the role of miRs in modulating inflammatory processes, cell signaling, differentiation, homeostasis,[18,19] and controlling intracellular bacterial and viral infections,[28,39] but there is little information on genital chlamydial infection. A PCR array (Figure S4) containing 88 miRs reported to contribute to immunopathological processes in several disease models[40] and regulated in the female reproductive tract[22] was used to

identify putative miRs associated with *C. muridarum* infection. MiR profiling in the lower and upper regions[9,14,31,32] of the murine genital tract was evaluated (Fig. 1) at 6 and 12 days post-*i. vag C. muridarum* infection (5×10^4 IFU) in C57BL6 mice. Bacterial burden at day 6 following *C. muridarum* challenge (307,300 ± 121,700 IFU) was reduced by day 12 (12,700 ± 8900 IFU) post-challenge with resolution of vaginal infection by day 30 post-challenge (Fig. 2). As shown in Fig. 1, 77 of the 88 miRs (>80%) associated with inflammation and immunopathology were downregulated (green rectangles and dots in Fig. 1a) at day 6 in the LGT of *C. muridarum*-challenged mice compared to mock-infected control mice. In contrast, a greater proportion (≅45%) of UGT associated miRs remained unchanged (Fig. 1b, black rectangles and dots). At day 12 post-infection, more than 40% of the miRs were upregulated in the LGT (Fig. 1c, red rectangles and dots), while less than 15% and approximately 35% of the miRs were upregulated or unaffected, respectively in the UGT (Fig. 1d). Overall, regulation of immunopathology related miRs was greater in LGT than UGT during first 12 days of infection with initial downregulation (day 6) followed by upregulation at day 12. Eleven of the 88 miRs ($P < 0.05$) exhibiting greater than ±2-fold change following *C. muridarum* challenge compared to controls were selected for further study (Table I). Importantly, four of these 11 miRs (miR-125b, -16, -23b, 182) are abundant in the female reproductive tract.[22] These 11 miRs are referred to as 'infection associated' and modulated in a spatial (lower versus upper) and temporal (day 6 versus day 12) fashion following

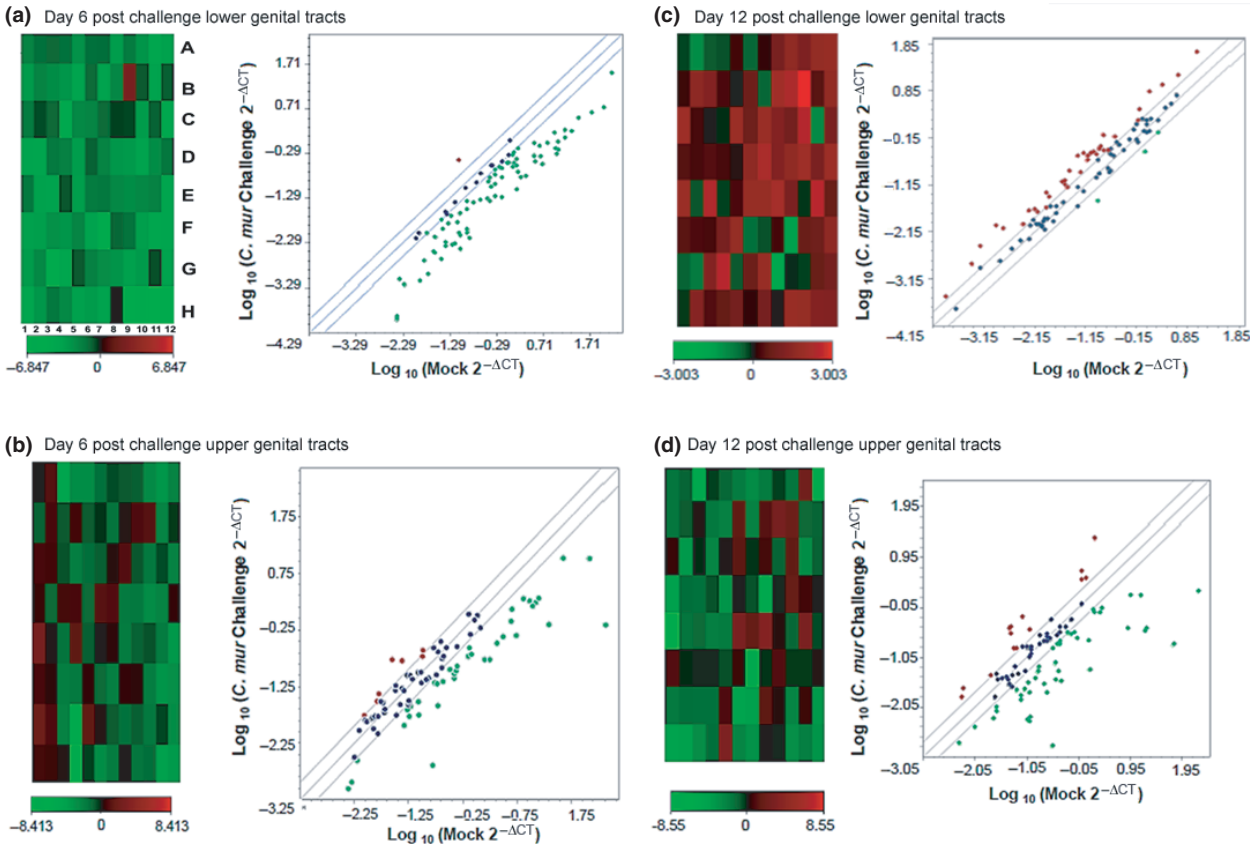

Fig. 1 *Chlamydia muridarum* Infection Regulates Host microRNA Expression. C57BL/6 mice were challenged intravaginally with 5×10^4 IFU. The genital tract was excised at days 6 (a, b) and 12 (c, d) post-infection and designated lower (a, c) and upper (b, d), respectively. RNA was extracted, converted to cDNA, and real-time PCR was performed by loading samples on 'Inflammation and Immunopathology' focused RT^2 miRNA plates. Differential miRNA heat map profiles and corresponding scatterplot analyses were plotted by comparing miRNA profiles of *C. muridarum*-infected to mock-infected control mice at indicated time points. Individual miR was presented as a rectangle and a dot in the heat map and scatter plot, respectively (n = 3/time point).

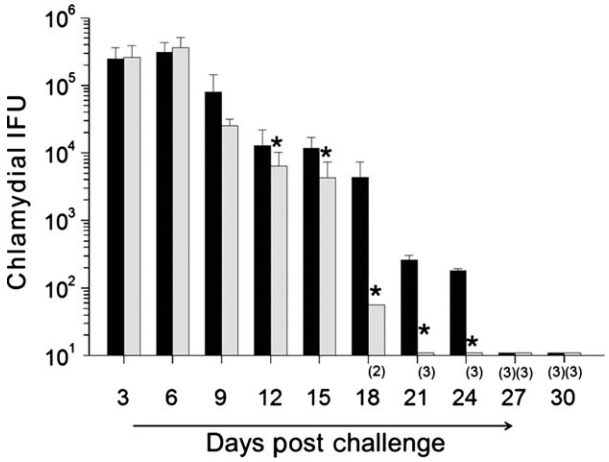

Fig. 2 Bacterial Burden in Mock-Vaccinated and rCPAF-Vaccinated Mice Following *Chlamydia muridarum* Infection. Resolution of *C. muridarum* genital infection in mock-vaccinated *C. muridarum*-challenged (Black Bars) and rCPAF-vaccinated *C. muridarum*-challenged (Gray Bars) C57BL/6 WT mice ($n = 3$). Mice were challenged *i.vag* with 5×10^4 IFU, and vaginal chlamydial shedding was monitored at 3-day intervals. Each bar represents the mean ± S.D. chlamydial IFU recovered from vaginal swabs at the indicated days following genital challenge. Significant difference in numbers of recovered chlamydial organisms from mock vaccinated compared to those of rCPAF-vaccinated WT (*$P < 0.05$). Numbers in parenthesis on the *X*-axis represent number of mice that resolved infection.

C. muridarum challenge (Table I). These 11 infection-associated miRs were not significantly regulated in a spatiotemporal fashion in rCPAF-vaccinated mice compared to mock-vaccinated (*i.e., C. muridarum* challenge) mice at day 6 and 12 post-challenge (Figure S1 and Table S1). Furthermore, miR expression analysis using miR-specific PCR primers confirmed nine of the 11 miRs changed greater than ±2-fold at 6 days post-infection with 5×10^4 IFU *C. muridarum* in the LGT (Fig. 3) with the exception of miR-16 and -451 (Table I). Increased *C. muridarum* challenge dose 5×10^5 and 5×10^6 previously reported to increase immune response[41,42] did not significantly alter expression of 6 (miR- 135a, 183, 182, 30e, 23 and 30c) of the nine infection specific miRs. Although significant differences in the fold change of miR-146 and -125b were observed following increased challenge dose, the trend of regulation (upregulated for miR-146a and downregulated for 125b compared to mock-challenged controls, respectively) was not consistent with increasing challenge doses (Fig. 3). Because no overall difference in miR modulation was observed with increasing infection dose previously shown to increase immune response,[41,42] an infection dose of 5×10^4 IFU was used for all subsequent analyses. These 9 miRs have been previously reported to contribute to critical functions including initiation of innate immune signaling and controlling inflammatory cytokines;[17,18,27,43,44] their role in genital CT infection remains to be elucidated. To this end, knockdown of miR-125b, -30c, and -182 in HeLa cells with miR-specific inhibitors (with >80% transfection knockdown efficiency), resulted in significant increase in chlamydial IFU compared to control (Fig. 4a) and modulation of NF-κβ activation and IRF-1 levels (Fig. 4b), suggests these miRs may affect chlamydial infectivity and inflammatory gene function. Significant reduction in NF-κβ activation was detected upon knockdown of miR-125b, -30c, and -182, respectively, whereas IRF-1 levels were significantly increased only upon miR-125b knockdown (Fig. 4b). STAT-1 levels were not affected by knockdown of miR-125b, -30c, and -182 (Fig. 4b).

C. muridarum Immune Responses Contribute to Host microRNA Responses

Our laboratory[33,34,45] and others[8,9,15] have shown that antigen-specific $CD4^+$ T cells and IFN-γ provide robust protection in primary infections and are essential for vaccination-generated protection against chlamydial infection. Antigen-specific $CD8^+$ T cells have been reported to contribute to immune responses[46,47] as well as development of pathology.[12] To investigate the contribution of $CD4^+$ and $CD8^+$ T cells as well as interferon-γ in modulating these nine miRs at the site of infection, we compared miR expression in WT mice to immunodeficient mice 6 days post-challenge. As shown in Fig. 5, significant differences in expression of mir-125b-5p, -135a, -183, and -182 in $CD4^{-/-}$ mice (white bars) compared to WT mice challenged with *C. muridarum* were observed suggesting that $CD4^+$T cells may contribute to modulation of infection-associated miR response at the infection site. In contrast, there were no significant differences in expression of these miRs in *C. muridarum*-infected IFN-$\gamma R^{-/-}$ (Figure S2A) and $CD8^{-/-}$ (Figure S2B) mice compared to WT LGT at the same time (day 6). Importantly, no statistically significant differences in expression of these miRs in the LGT of mock-infected WT, CD4, CD8, and IFN-γ knockout mice (data not shown).

Table I *Chlamydia muridarum* Genital Infection Modulates Host microRNA Responses in a Spatiotemporal Fashion

C. muridarum Infection								
	Day 6				Day 12			
	Lower GT		Upper GT		Lower GT		Upper GT	
miR	Fold Change	*P* value	Fold Change	*P* value	Fold Change	*P* value	Fold Change	*P* value
125b-5p	−17.35	0.015	−11.31	0.131	+3.23	0.372	+4.66	0.328
135a	−13.89	0.034	−1.77	0.85	−1.03	0.964	−4.60	0.367
16	−8.87	0.014	−3.30	0.15	+5.63	0.325	+2.80	0.34
214	−4.30	0.049	−1.59	0.435	−1.09	0.582	−2.23	0.47
30c	−19.16	0.036	−10.21	0.122	+1.71	0.372	−1.28	0.37
30e	−16.76	0.01	−8.24	0.124	+3.00	0.31	+1.07	0.54
182	−19.29	0.004	−4.80	0.431	+2.05	0.363	−1.98	0.46
183	−14.76	0.022	+1.32	0.60	+1.31	0.43	−21.06	0.20
23b	−17.19	0.01	−11.63	0.128	+1.58	0.41	−1.45	0.36
146	+3.60	0.01	+2.10	0.28	+3.95	0.29	+1.33	0.60
451	+6.10	0.03	−1.40	0.64	+1.57	0.38	+1.39	0.79

C. muridarum-Induced Host microRNAs Target Structural and Stress Proteins at the Infection Site

MicroRNAs have been reported to modulate cellular functions and immune responses by regulation of gene products.[17,18,27,28,48] To correlate host miR regulation with host protein expression following *C. muridarum* challenge, we conducted proteomic analyses to identify differentially expressed proteins at day 6 in the LGT of mock- and *C. muridarum*-challenged mice. We observed significant regulation of acute phase response signaling, structural reorganization, and stress-/inflammation-related proteins in *C. muridarum*-challenged mice (Figure S3). Among the differentially expressed proteins, we observed two proteins, HSPB1 (heat-shock protein B1) and AHSG (alpha-2HS-glycoprotein or fetuin-1 contains predicted binding sites for miR-135a and -182 (Fig. 6a, *in silico* prediction using www.microRNA.org). Interestingly, miR-135a was predicted to have binding sites in both HSPB1 and AHSG, suggesting the robustness and redundancy of miR-protein modulation (Fig. 6a). These two proteins were significantly upregulated in *C. muridarum*-infected WT compared to mock-infected WT mice and correlated with miR-135a and -182 expression which was downregulated following *C. muridarum* challenge (Fig. 1a and Table I). Additionally, these two proteins were significantly downregulated in $CD4^{-/-}$ mice compared to WT following infection (Fig. 6b,c), and these changes correlated to significant upregulated expression of miR-135a and -182 (Fig. 5). We next mapped, that is, identified the 'interactome' network for known protein–protein interaction of *C. muridarum* infection-modulated proteins by mass spectrometry. These IPA analyses revealed HSPB1 and fibronectin 1 (FN1) ($P = 2.3 \times 10^{-2}$) and AHSG and decorin (DCN) ($P = 8.59 \times 10^{-8}$) to interact significantly with each other (data not shown). Comparative protein expression assays indicated that both FN1 and DCN were downregulated upon chlamydial infection in WT but not $CD4^{-/-}$ mice (Fig. 7) consistent with IPA-predicted protein networking with HSPB1 and AHSG, respectively. As shown in Fig. 7a, FN1 levels were significantly reduced in WT mice compared to mock-infected WT or *C. muridarum*-infected $CD4^{-/-}$ mice. Fibronectin binding proteins have been reported to interact with HSP family proteins.[49] FN-Integrin signaling is associated with HSP induction and reduced apoptosis in gut injury.[50] Fibronectin has been reported to interact with bacterial proteins and be involved in bacterial pathogenesis.[51–53] Fibronectin is known to be recruited by *C. trachomatis* serovar L2 and D EBs to facilitate tissue colonization,[54] but its role in bacterial pathogenesis is not known. We also observed DCN (Fig. 7b), to be regulated in a manner opposite to its interacting member AHSG (Fig. 6c) consistent with a previous report on DCN–AHSG interaction.[55] Decorin has been indicated previously in microbial adhesion[56]

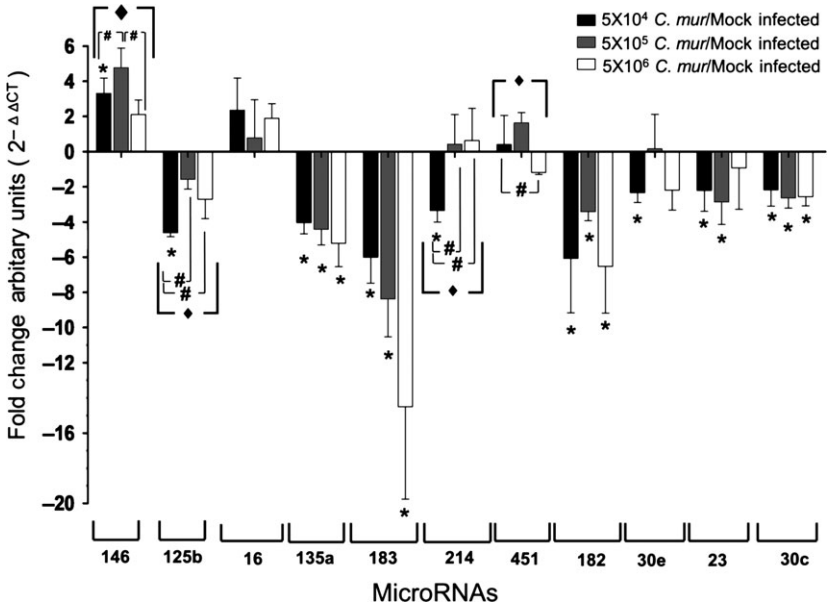

Fig. 3 Increasing Dose *Chlamydia muridarum* Modulate Infection-associated miRs Expression. Increased inoculating dose of *C. muridarum* differentially modulates miRs but not significantly. Fold changes in miR expression were calculated based on differences and plotted as ratio of miR expression in 5×10^4 *C. muridarum*/mock infected (black bars); or 5×10^5 *C. muridarum*/mock infected (gray bars) or 5×10^6 *C. muridarum*/mock infected (white bars). All miR expression analyses were normalized to RNU6 or SNORD68 expression values and calculated using RT^2 profiler PCR Array Data Analysis version 3.5 (Qiagen). Two-tailed Student's *t*-test was used for statistical analyses between test and control groups. *$P < 0.05$. *X*-axis represents microRNAs in 5×10^4 *C. muridarum*/mock infected; 5×10^5 *C. muridarum*/mock infected; 5×10^6 *C. muridarum*/mock infected; # $P < 0.05$ intragroup comparison between black, gray and white bars; ♦ ANOVA with Tukey B correction.

and contribute to bacterial pathogenesis.[57] *Chlamydia pneumoniae* EB adhesion and infectivity are affected by interactions between invasin protein Pmp21, and human epidermal growth factor receptor[58] known to have decorin binding sites.[59] Collectively, these results suggest that *C. muridarum* infection-induced host response (protein expression) may be functionally related to *C. muridarum* infection-associated miRs. However, their specific contribution to bacterial pathogenesis warrants further investigation.

Discussion

We provide evidence suggesting (i) modulation of host microRNAs following *in vivo C. muridarum* genital infection and (ii) their possible contribution to infectivity and associated anti-chlamydial immunity. Specifically, expression of host miRs (Fig. 1) was downregulated in the genital tract of infected mice compared to mock controls at day 6 post-bacterial challenge. We also observed that these miRs were significantly regulated in a time-dependent fashion (day 6 compared to day 12) in the LGT (Table I). In a similar fashion, we ascertained (Figure S1) if these infection-associated miRs were regulated following intranasal vaccination using rCPAF (recombinant chlamydial protease-like activity factor, serovar L2, a protective chlamydial antigen[35] reported by our laboratory to provide protection against genital *C. muridarum* challenge.[36] We observed no significant regulation of infection-associated miRs (Table S1) following rCPAF vaccination at days 6 and 12 post-challenge in the LGT and UGT suggesting these 11 infection-associated miRs are not modulated in rCPAF vaccination (Figure S1, Table S1, and Fig. 2) and may be associated only with primary infection and not with rCPAF vaccine.

The spatiotemporal modulation of infection-associated miRs (Table I, Fig. 1) suggesting these miRs may be associated with bacterial infectivity at day 6 were corroborated by knockdown of miR 125b-5p, -30c, and -182 in host cells which resulted in an increase of *C. muridarum* IFU. These *in vitro* observations partially support the *in vivo* findings and suggest a possible involvement of these miRs in modulation of chlamydial infection and colonization.

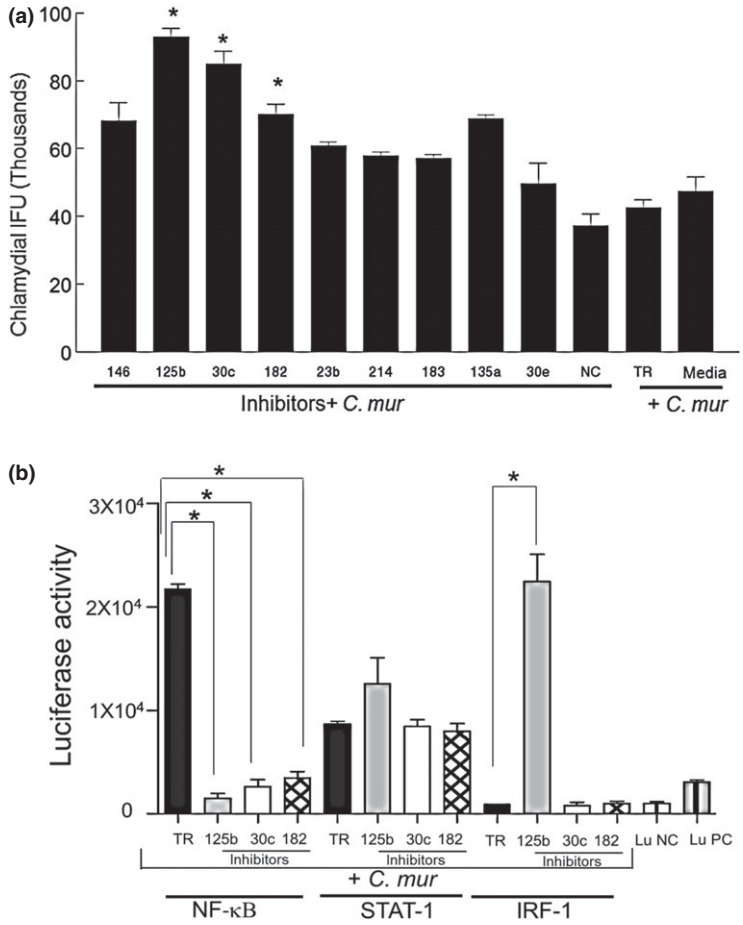

Fig. 4 *Chlamydia muridarum* Infectivity is Modulated by Infection-associated miRs. HeLa 229 cells were seeded at a density of 2.5×10^5 and transfected with either (a) miR-specific inhibitors (miScript miRNA inhibitors; Qiagen) or (b) co-transfected with luciferase tagged reporter constructs for either NF-κB, STAT-1 or IRF-1 (CIGNAL Assay; Qiagen). (a) Cells were infected with 0.1 MOI *C. muridarum* EBs, and infectivity was measured by enumerating IFU at 24 hr post-infection. Effect of miR-specific inhibitors on *C. muridarum* infectivity was calculated as the difference between IFU counts in inhibitor-transfected wells and wells with *C. muridarum* alone (Media). Wells containing NC-non-specific miR inhibitor or TR-transfection reagent (Attractene; Qiagen) served as controls. (b) Luciferase activity was measured for activation of NF-κB (6 hr post-infection), STAT-1 or IRF-1 (24 hr post-infection). Activation of inflammatory pathways was calculated as the difference in luciferase activity between *C. muridarum*-infected wells transfected with miR inhibitors and transfection reagent (TR) alone. Wells containing LuNC- Luciferase Negative Control or LuPC- luciferase-positive control served as assay controls. Student's *t*-test was used for statistical analyses between test and control groups. $*P < 0.05$.

Furthermore, under miR -125b-5p, -30c, and -182 knockdown conditions, we investigated modulation of inflammatory signaling molecules, that is, NF-κB, IRF-1, and STAT-1 previously reported to be involved in chlamydial infection and control.[60–65] Using a luciferase reporter assay, we observed *C. muridarum* infection under miR-125b-5p, -30c, and -182 knockdown conditions (Fig. 4a) resulted in significant decrease of NF-κB (Fig. 4b). MiR-125b and miR-125a knockdown has been reported to downregulate NF-κB activity following tumor necrosis factor alpha-induced protein 3 targeting.[66] Additionally, comparative profiling of infection-associated miRs revealed miR-125b and -182 along with -183 and -135a to be significantly upregulated in *C. muridarum*-infected $CD4^{-/-}$ mice compared to WT (Fig. 5). Importantly, mir-125b has been reported to inhibit T-cell differentiation by controlling *IFNG, IL2RB, and PRDM1* genes.[27] MiR-125b has also been shown to increase macrophage activation, responsiveness to IFN-γ,[43] promote cell survival *via* NF-κB[67], and regulate MyD88[68] (a critical molecule

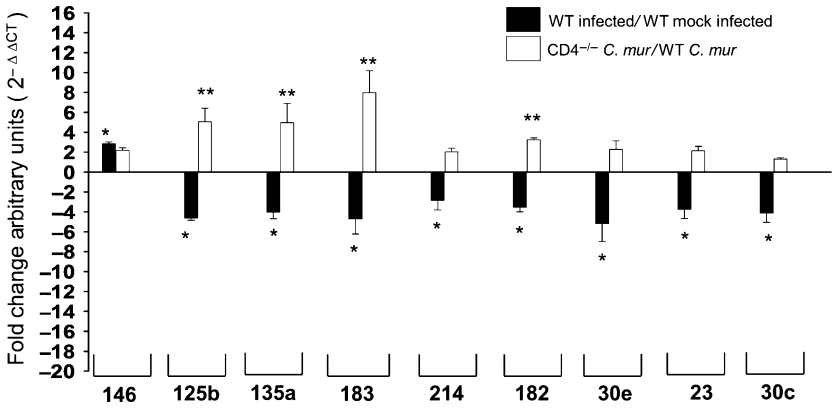

Fig. 5 Host Immune Response Against *Chlamydia muridarum* Contributes to Modulation of microRNAs. C57BL/6 (WT) and CD4$^{-/-}$ mice (n = 3) were infected with 5 × 10^4 IFU and miR expression in the lower genital tract (LGT) assessed at day 6 post-infection. Two-tailed Student's t-test was used for statistical analyses between test and control groups. *P < 0.05 for *C. muridarum*-infected WT compared to mock-infected WT, **P < 0.05 for *C. muridarum*-infected CD4$^{-/-}$ compared to *C. muridarum*-infected WT.

involved in signaling cascades leading to NF-κB-mediated transcription of proinflammatory genes[69]). Moreover, mir-182 which belongs to the miR-183, -96, -182 cluster[70] has been reported to upregulate NF-κB in malignant gliomas[71] and is induced by IL-2 and shown to clonally expand helper T cells.[72]

Similar to NF-κB, IRF and STAT family have been reported to be involved in downstream signaling involving cytokine production.[73,74] Significantly increased IRF-1 expression was observed following miR-125b inhibition (Fig. 4b).[63,64] There is to date no evidence for miR-125b influence on IRF-1 production. Additionally, miR-125 has been reported to be a non-responsive regulator of interferon-β[75] and may not directly affect IRF-1-mediated chlamydial reduction involving interferon-β. In contrast, knockdown of miR-30c and -182 did not affect IRF-1 (Fig. 4b). We observed no significant change in relative levels of STAT-1 expression under miR-125b-5p, -30c, or -182 knockdown conditions compared to *C. muridarum*-infected HeLa cells suggesting STAT-1 may not be associated with these miRs and *C. muridarum* infectivity. A recent report[76] suggests antigen-specific CD4$^+$ T cells and expression of MyD88 are required for resolution of genital *C. muridarum* infection. Furthermore, interferon regulation[63,64] and generation of antigen-specific CD4$^+$ Th1 responses which control bacterial infection are well documented.[8,14,16] Taken together, these results suggest that 'infection-associated' miRs may contribute to chlamydial invasion and modulate inflammatory pathways.

Data from our *in vitro* experiments reported here (Fig. 4) provide evidence for differential production/expression of inflammatory molecules (NF-κB, and IRF-1) following *C. muridarum* infection under miR knockdown conditions (miR-125b-5p, -30c, and -182). However, further investigation using stimulators/agonists of NF-κB, IRF-1, and STAT-1 (which was not significantly regulated under miR-125b-5p, -30c, and -182 knockdown conditions, Fig. 4b) in miR-125b-5p, -30c, and -182 deficient HeLa cell lines will provide needed insight into downstream pathways that contribute to *C. muridarum* infectivity. In miR-125b-5p, -30c, and -182 deficient HeLa cell lines will provide needed insight into downstream pathways that contribute to *C. muridarum* infectivity. Importantly, it must be appreciated that (i) *in vitro* experiments using miR-specific inhibitors were carried out in HeLa cells (a human cell line), and (ii) murine and human chlamydial genital infection models are dissimilar in certain immunologic aspects.[3] Therefore, further investigation of miR regulation of inflammatory molecules and development of adaptive immunity 'network' will be greatly strengthened by the availability of specific miR-deficient mice.

We next determined cellular targets for infection-associated miRs. Mass spectrometry analyses (Figure S1) revealed regulation of structural and stress proteins involved in signaling, cellular actin reorganization, and antiapoptotic mechanisms.[77–80] Among these *C. muridarum* infection-modulated proteins, we observed probable regulation of two proteins, that is, HSPB1 and AHSG by infection-associated miRs (Fig. 6b,c) corroborating our *in silico* prediction (Fig. 6a). HSPB1 or HSP27 (Fig. 6b) belongs to a family of proteins reported to play a crucial role in

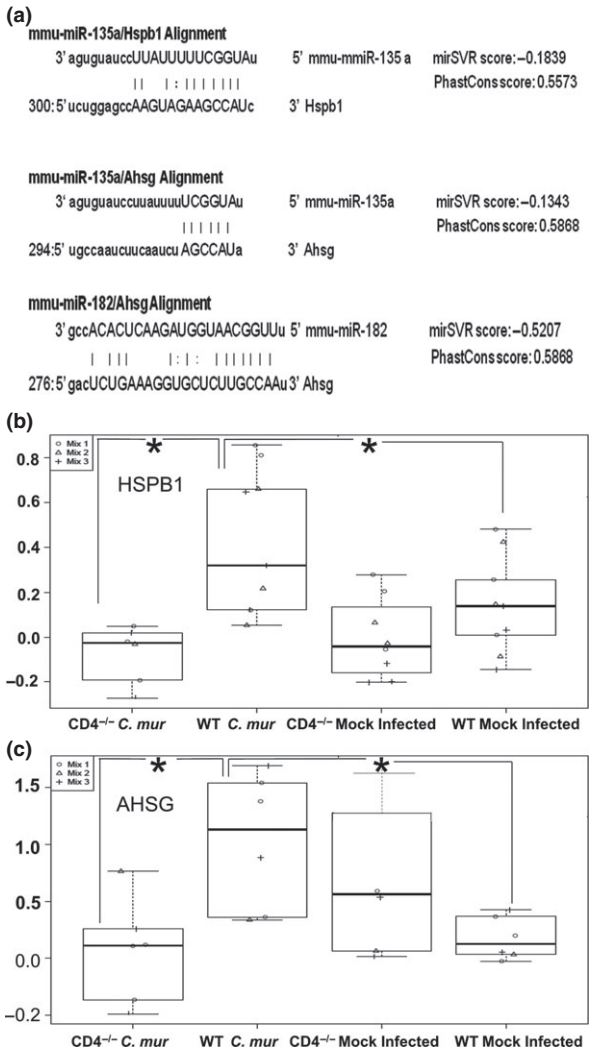

Fig. 6 *Chlamydia muridarum* Infection-induced microRNAs Regulate Structural and Stress proteins at Day 6 Post-Infection. C57BL6 (WT) and CD4$^{-/-}$ mice (n = 3) were infected with 5 × 10^4 IFU, and protein profiling by mass spectrometry was performed at day 6 LGT as previously described under Methods. (a) Bioinformatic analysis of putative binding sites in genes encoding proteins found to be modulated by *C. muridarum* infection-associated miRs was performed using miR target predictive algorithm (microRNA.org). Protein production of (b) HSPB1 and (c) AHSG was normalized to pooled reference controls and plotted as differences in all experimental groups at day 6 in the LGT. Student's t-test was used for statistical analyses between test and control groups. *P < 0.05. HSPB1, heat-shock protein B1; AHSG, alpha-2HS-glycoprotein.

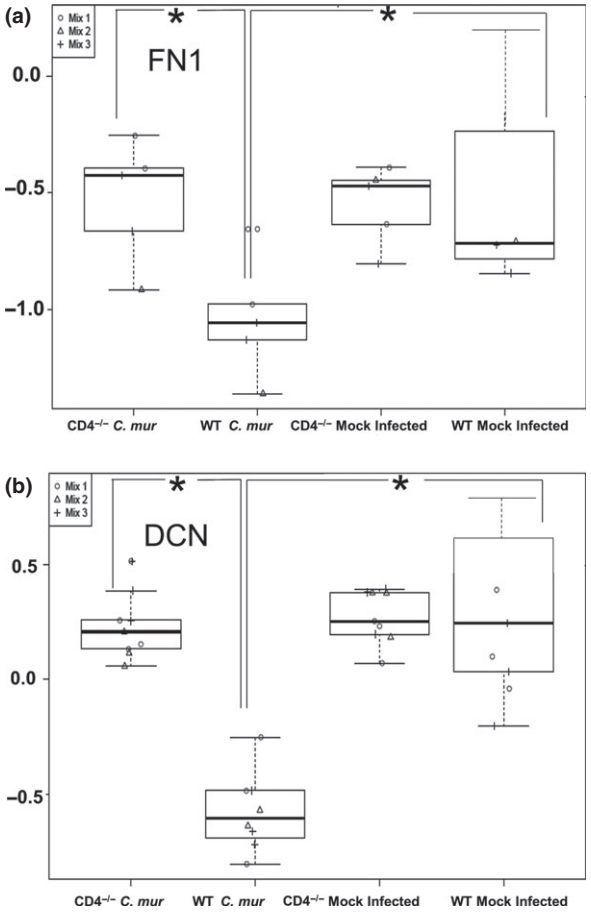

Fig. 7 *Chlamydia muridarum* Infection-associated microRNAs Regulated Proteins Influence Modulation of Other Proteins. Protein profiling by mass spectrometry was performed at day 6 in the lower genital tract as previously described under Methods. Fold change in protein production was normalized to pooled reference controls and plotted as differences in all experimental groups at day 6 in the LGT. Student's t-test was used for statistical analyses between test and control groups. *P < 0.05. FN1-Fibronectin 1; DCN-Decorin.

maintaining integrity and function in several cell types.[77] Additionally, this protein family has been reported to initiate adaptive immune responses and exert immunoregulatory functions.[78,79] The phosphorylation of HSP27 and associated inhibition of chlamydial growth by rottlerin involving p38-regulated/activated protein kinase have recently been reported.[81] AHSG is reported to be associated with inflammatory processes[80] and has been shown to be produced in increased levels in women diagnosed with endometriosis.[82,83] Additionally, miR-135a and -182 which have binding sites for these two proteins (Fig. 6a) have been reported to be functionally active in women with endometriosis[84] or endometrial adenocarcinoma.[85] MiR-135a and -182 were found to be significantly regulated in *C. muridarum*-infected CD4$^{-/-}$ mice compared to WT animals (Fig. 5) suggesting a probable link between these miRs, their respective targets, and the contribution

of $CD4^+$ T cells in the *C. muridarum*-infected genital tract.

Data described in this report are consistent with the recent findings of Igietseme et al.,[30] indicating miR-induced response in UGT pathology following chlamydial infection. Results presented here focus at day 6 post-challenge and miRs that may influence bacterial colonization. Recently, the contribution of miRs in ocular CT infection has been reported[29] indicating miR-147b and -1285 to be upregulated in patients with inflammatory trachomatous scarring. Interestingly, miR-23b and -30c were also found to be modulated in patients with conjunctival scarring with or without inflammation compared to healthy individuals suggesting the ubiquitous nature of these two miRs in chlamydial infections. Additionally, the contribution of miRs in the lungs of neonatal mice infected with CT also has recently been described[86] indicating antagomiRs of selected miRs regulated upon CT infection in neonates prevented development of serious respiratory sequelae similar to reports from our laboratory.[87] In summary, this study corroborates further the importance of host miR regulation in chlamydial infections[29,30,86,88,89] and underscores the need for future studies on the underlying molecular mechanism(s) of pathogenesis.

Author's contributions

RG carried out the experiments. TA and SW assisted with *in vitro* experiments, bacterial inoculum preparation, and real-time microRNA PCR amplifications. RG and WEH performed the proteomic analyses. RG, NG, and BPA contributed to the conceptual study design and critical analysis. RG, JY, JPC, AKM, SAB, and BPA analyzed the data, drafted, and edited the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. rCPAF Vaccination Modulates Host microRNAs Following *C. muridarum* Infection.

Figure S2. Host Immune Responses Against *C. muridarum* Contribute to Modulation of microRNAs.

Figure S3. Functional Distribution of Protein–Protein Interaction Networks.

Figure S4. Plate layout of 88 microRNAs contributing to Inflammation and Immunopathology.

Table S1. rCPAF Vaccination Does Not Affect Spatiotemporal Modulation of Infection-associated MicroRNAs.