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

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Abstract

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

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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.

revealed regulation of miR-135a and -182 and associated proteins *i.e.*, 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.

Keywords

Chlamydia muridarum; host responses; microRNAs; murine genital tract

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. In order 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. mur*), 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 sequelae⁸. Intravaginal (*i. vag.*) inoculation of *C. mur* 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. mur* 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, *i.e.* 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 scarring²⁹. Recently, Igietseme *et al.* reported the role of host caspases in tissue apoptosis and associated genital chlamydial pathogenesis³⁰. Importantly, these investigators provide evidence on inactivation of DICER and involvement of miRs that regulate growth and differentiation in CT infected mice³⁰.

Given that immunological events¹⁰ occurring relatively early in genital chlamydial infection lead to inflammation and may influence the development of upper genital pathology¹⁵, 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 (Supp. Fig. 4) 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. mur* challenge. Comparative profiling revealed 9 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. mur* numbers in 3 of these 9 miRs *i.e.* -125b-5p, -30c, and -182. Additionally, following *C. mur* infection, significant regulation of inflammatory molecules, *i.e.*, NF- κ B (miR-125b-5p, -30c, and -182 knockdown conditions), and IRF-1 levels (miR-125b-5p knockdown conditions) was 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 9 miRs in WT and CD4^{-/-} animals’ LGT isolated at day 6 post *C. mur* 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 α 2HS-glycoprotein (AHSG) by miR-135a, -182 following *C. mur* 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 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

C. muridarum was grown on confluent Hela cell monolayers and purified as previously described³³. Briefly, infected Hela cells were lysed using a sonicator (40 V pulses for 10 s on ice; Fisher, Pittsburgh, PA), 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 (SPG) buffer until used.

Mice

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

Intranasal Immunization procedure

C57BL6 WT mice were immunized as described previously³⁴. Specifically, groups of mice were anesthetized (3% isoflurane) and immunized intranasally on day 0 with 15 μ g of rCPAF in 25 μ l sterile PBS. On days -1, 0, and +1 10 μ g 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 μ g/mouse) provided optimal protection against genital *C. mur* challenge³³.

Intravaginal Infection

In order 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., NY) on day 5 before infection³⁵. Mice were inoculated *i.vag.* with 5×10^4 *C. mur* 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. mur* challenge and subsequent plating on Hela cell monolayers grown on culture coverslips³⁶. 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 5 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 group³⁴.

RNA Isolation and miRNA PCRs

At the indicated days following infection, mice were euthanized and the genital tract dissected out, cut into two sections, *i.e.* 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) according to manufacturer's instructions. Total RNA was assessed using a Nanodrop Spectrophotometer (ThermoScientific, Asheville, NC) and RNA samples (1 μ g with A_{260/280} and A_{260/230} values of ≈ 2.0 and ≈ 1.8 or higher, respectively) were converted to cDNA using a RT² 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 RT² miRNA plates (MAM 104D, Qiagen) using a RT² SYBR Green qPCR Mastermix per manufacturer's instructions (Qiagen) and BioRad CFX96 thermocycler (BioRad, Hercules, CA). 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). All miR expression analyses were normalized to housekeeping RNU6-2_1 or SNORD68 expression values and determined using $2^{(-\text{Average CT})}$ 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) and per manufacturer's specifications, transfection efficiency and knockdown was observed to be >80%. At 16 h post transfection, cells were infected with 0.1 MOI *C. mur* EBs. Infected cells were fixed 24 h 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. mur* IFU infected wells and wells with miR-specific inhibitors.

***In vitro* CIGNAL Assay**

To monitor modulation of Nuclear Factor *i.e* NF- κ B, and the Signal Transducer and Activator of Transcription *i.e* STAT-1, and Interferon Regulatory Factor *i.e* IRF-1 following *C. mur* infection in miR knockdown conditions, Dual luciferase/renilla activity for NF- κ B, 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 h post transfection, these transiently transfected cells were infected with 0.1 MOI *C. muridarum* EBs. NF- κ B (6 h post infection), STAT-1, and IRF-1 (24 h post infection) activity were determined by monitoring luciferase/renilla levels using the Dual Glo Luciferase assay (Promega, Madison, WI) according to manufacturer's instructions and a Synergy 2 Multi-Mode Microplate Reader (Biotek, Winooski, VT).

Mass Spectrometry

For analysis of putative downselected miR- targets, mass spectrometry was performed on genital tract tissue from day 6 naïve, *C. mur* infected WT, and CD4^{-/-} mice. Total cell protein extraction was accomplished using RIPA Lysis Buffer Kit (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer's protocol. Protein concentration was determined using an Invitrogen EZQ Protein Quantitation Kit (Invitrogen, Grand Island, NY). Quantitative tandem mass spectrometry-based "microwave & magnetic (M²)" 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 *i.e* 127–130 while pooled reference materials were encoded with TMT reagents 126 or 131 (*C. mur* infected CD4^{-/-} and infected WT, respectively). To normalize across all

specimens, TMT-encoded cell lysate were 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). All experimental results are calculated on the mean $\pm$ SEM of two to five independent experiments. GraphPad Prism 5 (La Jolla, CA) was used to perform all tests of significance. MiR analysis was performed using RT² 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 (Suppl. Fig. 4) containing 88 miRs reported to contribute to immuno-pathological processes in several disease models⁴⁰ and regulated in the female reproductive tract²² was used to identify putative miRs associated with *C. mur* infection. MiR profiling in the lower and upper regions^{9, 14, 31, 32} of the murine genital tract were evaluated (Fig. 1) at 6 and 12 days post *i. vag C. mur* infection (5×10^4 IFU) in C57BL6 mice. Bacterial burden at day 6 following *C. mur* challenge (307300 ± 121700 IFU) were reduced by day 12 (12700 ± 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. mur* challenged mice compared to mock infected control mice. In contrast, a greater proportion ($\approx 45\%$) 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. mur* challenge compared to controls were selected for further study (Table 1). Importantly, 4 of these 11 miRs (miR-125b, -16, -23b, 182) are abundant in the female reproductive tract²². These 11 miRs are referred to as 'infection associated' and modulated in a spatial (lower vs. upper) and temporal (day 6 vs. day 12) fashion following *C. mur* challenge (Table 1). These 11 infection associated miRs were not significantly regulated in a spatio-temporal fashion in rCPAF vaccinated mice compared to mock vaccinated (*i.e C. mur* challenge) mice at day 6

and 12 post challenge (Supp Fig. 1 and Supp Table 1). Furthermore, miR expression analysis using miR-specific PCR primers confirmed 9 of the 11 miRs changed greater than ± 2 fold at 6 days post-infection with 5×10^4 IFU *C. mur* in the LGT (Fig. 3) with the exception of miR-16 and -451 (Table 1). Increased *C. mur* challenge dose 5×10^5 and 5×10^6 previously reported to elevate immune response^{41, 42}, did not significantly alter expression of 6 (miR- 135a, 183, 182, 30e, 23 and 30c) of the 9 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 elevate 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- κ B activation and IRF-1 levels (Fig. 4B) suggests these miRs may affect chlamydial infectivity and inflammatory gene function. Significant reduction in NF- κ B 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 anti-chlamydial immune responses^{46, 47} as well as development of pathology¹². To investigate the contribution of CD4⁺ and CD8⁺ T cells as well as interferon- γ in modulating these 9 miRs at the site of infection, we compared miR expression in WT mice to immuno-deficient 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. mur* was 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 difference in expression of these miRs in *C. mur* infected IFN- γ R^{-/-} (Supp. Fig. 2A) and CD8^{-/-} (Supp. Fig 2B) 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).

***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}. In order to correlate host miR regulation with

host protein expression following *C. mur* challenge, we conducted proteomic analyses to identify differentially expressed proteins at day 6 in the LGT of mock and *C. mur* challenged mice. We observed significant regulation of acute phase response signaling, structural reorganization, and stress/inflammation related proteins in *C. mur* challenged mice (Supp. Fig. 3). Among the differentially expressed proteins, we observed two proteins, HSPB1 (Heat Shock Protein B1) and AHSG (Alpha-2HS-Glycoprotein, or Fetuin-1 contain 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. mur* infected WT compared to mock infected WT mice, and correlated with miR-135a, and -182 expression which was downregulated following *C. mur* challenge (Fig. 1A and Table 1). 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 *i.e* identified the “interactome” network for known protein-protein interaction of *C. mur* 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. mur* infected CD4^{-/-} mice. Fibronectin binding proteins have been reported to interact with HSP family proteins⁴⁹. FN-Integrin signaling is associated with HSP induction and reduced apoptosis in gut injury⁵⁰. 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 colonization⁵⁴, 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 interaction⁵⁵. Decorin has been indicated previously in microbial adhesion⁵⁶, and contribute to bacterial pathogenesis⁵⁷. *Chlamydia pneumoniae* EB adhesion and infectivity is affected by interactions between Invasin Protein Pmp21, and human epidermal growth factor receptor⁵⁸ known to have decorin binding sites⁵⁹. 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 (1) modulation of host microRNAs following *in vivo* *C. muridarum* genital infection, and (2) their possible contribution to infectivity, and associated anti-chlamydial immunity. Specifically, expression of host miRs (Fig 1) were 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 1). In a similar fashion, we

ascertained (Supp Fig. 1) if these infection associated miRs were regulated following intranasal vaccination using rCPAF (recombinant chlamydial protease-like activity factor, serovar L2, a protective chlamydial antigen³⁵ reported by our laboratory to provide protection against genital *C. mur* challenge³⁶. We observed no significant regulation of infection associated miRs (Supp Table 1) 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 (Suppl Fig. 1, Suppl Table 1, and Fig. 2) and maybe associated only with primary infection and not with rCPAF vaccine.

The spatio-temporal modulation of infection associated miRs (Table 1, Fig. 1) suggesting these miRs may be associated with bacterial infectivity at day 6 was corroborated by knockdown of miR-125b-5p, -30c, and -182 in host cells which resulted in an increase of *C. mur* 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. Furthermore, under miR-125b-5p, -30c, and -182 knockdown conditions, we investigated modulation of inflammatory signaling molecules *i.e* NF- κ B, IRF-1, and STAT-1 previously reported to be involved in chlamydial infection and control^{60–65}. Using a luciferase reporter assay (Fig. 4A), we observed *C. mur* infection under miR-125b-5p, -30c, and -182 knockdown conditions resulted in significant decrease of NF- κ B (Fig. 4B). MiR-125b and miR-125a knockdown has been reported to downregulate NF- κ B activity following tumor necrosis factor, alpha-induced protein 3 targeting⁶⁶. Additionally, comparative profiling of infection associated miRs revealed miR-125b and -182 along with -183 and -135a to be significantly upregulated in *C. mur* 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* genes²⁷. MiR-125b has also been shown to increase macrophage activation, responsiveness to IFN- γ ⁴³, promote cell survival *via* NF- κ B⁶⁷, and regulate MyD88⁶⁸ (a critical molecule involved in signaling cascades leading to NF- κ B mediated transcription of proinflammatory genes⁶⁹). Moreover, miR-182 which belongs to the miR-183, -96, -182 cluster⁷⁰ has been reported to upregulate NF- κ B in malignant gliomas⁷¹, and is induced by IL-2 and shown to clonally expand helper T cells⁷².

Similar to NF- κ B, 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). Although increased IRF-1 levels lead to production of interferon which controls chlamydial infection^{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. mur* infected Hela cells suggesting STAT-1 may not be associated with these miRs and *C. mur* infectivity. A recent report⁷⁶, suggests antigen specific CD4⁺ T cells and expression of MyD88 is required for resolution of genital *C. mur* infection. Furthermore, interferon regulation^{95, 96} and generation of antigen specific CD4⁺ Th1 responses which control bacterial infection is 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- κ B, and IRF-1) following *C. mur* infection under miR knockdown conditions (miR-125b-5p, -30c, and -182). However, further investigation using stimulators/agonists of NF- κ B, IRF-1, and STAT-1 (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. mur* infectivity. Importantly, it must be appreciated that (1) *in vitro* experiments using miR specific inhibitors were carried out in Hela cells (a human cell line), and (2) murine and human chlamydial genital infection models are dissimilar in certain immunological aspects³. 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 (Supp Fig 1) revealed regulation of structural and stress proteins involved in signaling, cellular actin reorganization, and anti-apoptotic mechanisms^{77–80}. Amongst these *C. mur* infection modulated proteins, we observed probable regulation of two proteins, *i.e* HSPB1 and AHSG by infection associated miRs (Fig. 6B, C) corroborating our *in silico* prediction (Fig. 6A). HSPB1 or HSP27 (Fig. 6B) belong to a family of proteins reported to play a crucial role in maintaining integrity and function in several cell types⁷⁷. Additionally, this protein family has been reported to initiate adaptive immune responses, and exert immuno-regulatory functions^{78, 79}. The phosphorylation of HSP27 and associated inhibition of chlamydial growth by rottlerin involving p38-regulated/activated protein kinase (PRAK) has recently been reported⁸¹. AHSG is reported to be associated with inflammatory processes⁸⁰, and has been shown to be produced in elevated 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 endometriosis⁸⁴ or endometrial adenocarcinoma⁸⁵. MiR-135a and -182 were found to be significantly regulated in *C. mur* 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. mur* 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 reported²⁹ 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 described⁸⁶ indicating antagomiRs of selected miRs regulated upon CT infection in neonates prevented development of serious respiratory sequelae similar to

reports from our laboratory⁸⁷. In summary, this study^{29, 30, 86, 88, 89, 31, 87, 89, 90} 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Gerbase AC, Rowley JT, Mertens TE. Global epidemiology of sexually transmitted diseases. *Lancet*. 1998; 351(Suppl 3):2–4. [PubMed: 9652711]
2. Haggerty CL, Gottlieb SL, Taylor BD, Low N, Xu F, Ness RB. Risk of sequelae after *Chlamydia trachomatis* genital infection in women. *J Infect Dis*. 2010; 201(Suppl 2):S134–S155. [PubMed: 20470050]
3. Brunham RC, Rey-Ladino J. Immunology of *Chlamydia* infection: implications for a *Chlamydia trachomatis* vaccine. *Nat Rev Immunol*. 2005; 5:149–161. [PubMed: 15688042]
4. Simonetti AC, Melo JH, de Souza PR, Bruneska D, de Lima Filho JL. Immunological's host profile for HPV and *Chlamydia trachomatis*, a cervical cancer cofactor. *Microbes Infect*. 2009; 11:435–442. [PubMed: 19397882]
5. Centers for Disease Control and Prevention. Incidence, Prevalence, and Cost of Sexually Transmitted Infections in the United States. 2013. (<http://www.cdc.gov/std/stats/STI-Estimates-Fact-Sheet-Feb-2013.pdf>)
6. Hickey DK, Patel MV, Fahey JV, Wira CR. Innate and adaptive immunity at mucosal surfaces of the female reproductive tract: stratification and integration of immune protection against the transmission of sexually transmitted infections. *J Reprod Immunol*. 2011; 88:185–194. [PubMed: 21353708]
7. Wira CR, Veronese F. Mucosal immunity in the male and female reproductive tract and prevention of HIV transmission. *Am J Reprod Immunol*. 2011; 65:182–185. [PubMed: 21294802]
8. Morrison RP, Caldwell HD. Immunity to murine chlamydial genital infection. *Infect Immun*. 2002; 70:2741–2751. [PubMed: 12010958]
9. Farris CM, Morrison RP. Vaccination against *Chlamydia* genital infection utilizing the murine *C. muridarum* model. *Infect Immun*. 2011; 79:986–996. [PubMed: 21078844]
10. Darville T, Andrews CW Jr, Sikes JD, Fraley PL, Rank RG. Early local cytokine profiles in strains of mice with different outcomes from chlamydial genital tract infection. *Infect Immun*. 2001; 69:3556–3561. [PubMed: 11349013]
11. Rank RG, Lacy HM, Goodwin A, Sikes J, Whittimore J, Wyrick PB, Nagarajan UM. Host chemokine and cytokine response in the endocervix within the first developmental cycle of *Chlamydia muridarum*. *Infect Immun*. 2010; 78:536–544. [PubMed: 19841073]
12. Murthy AK, Li W, Chaganty BK, Kamalakaran S, Guentzel MN, Seshu J, Forsthuber TG, Zhong G, Arulanandam BP. Tumor necrosis factor alpha production from CD8⁺ T cells mediates oviduct pathological sequelae following primary genital *Chlamydia muridarum* infection. *Infect Immun*. 2011; 79:2928–2935. [PubMed: 21536799]
13. Rank RG, Whittimore J, Bowlin AK, Dessus-Babus S, Wyrick PB. *Chlamydiae* and polymorphonuclear leukocytes: unlikely allies in the spread of chlamydial infection. *FEMS Immunol Med Microbiol*. 2008; 54:104–113. [PubMed: 18657107]

14. Kelly KA. Cellular immunity and *Chlamydia* genital infection: induction, recruitment, and effector mechanisms. *Int Rev Immunol*. 2003; 22:3–41. [PubMed: 12710502]
15. Farris CM, Morrison SG, Morrison RP. CD4⁺ T cells and antibody are required for optimal major outer membrane protein vaccine-induced immunity to *Chlamydia muridarum* genital infection. *Infect Immun*. 2010; 78:4374–4383. [PubMed: 20660610]
16. Frazer LC, Scurlock AM, Zurenski MA, Riley MM, Mintus M, Pociask DA, Sullivan JE, Andrews CW Jr, Darville T. IL-23 induces IL-22 and IL-17 production in response to *Chlamydia muridarum* genital tract infection, but the absence of these cytokines does not influence disease pathogenesis. *Am J Reprod Immunol*. 2013; 70:472–484. [PubMed: 24238108]
17. Gantier MP, Sadler AJ, Williams BR. Fine-tuning of the innate immune response by microRNAs. *Immunol Cell Biol*. 2007; 85:458–462. [PubMed: 17621315]
18. Lindsay MA. microRNAs and the immune response. *Trends Immunol*. 2008; 29:343–351. [PubMed: 18515182]
19. Baumjohann D, Kageyama R, Clingan JM, Morar MM, Patel S, de Kouchkovsky D, Bannard O, Bluestone JA, Matloubian M, Ansel KM, Jeker LT. The microRNA cluster miR-17 approximately 92 promotes TFH cell differentiation and represses subset-inappropriate gene expression. *Nat Immunol*. 2013; 14:840–848. [PubMed: 23812098]
20. Maccani MA, Marsit CJ. Epigenetics in the placenta. *Am J Reprod Immunol*. 2009; 62:78–89. [PubMed: 19614624]
21. Sathe A, Patgaonkar MS, Bashir T, Reddy KV. MicroRNA Let-7f: A Novel Regulator of Innate Immune Response in Human Endocervical Cells. *Am J Reprod Immunol*. 2014; 71:137–153. [PubMed: 24405266]
22. Nothnick WB. The role of micro-RNAs in the female reproductive tract. *Reproduction*. 2012; 143:559–576. [PubMed: 22454533]
23. Shukla GC, Singh J, Barik S. MicroRNAs: Processing, Maturation, Target Recognition and Regulatory Functions. *Mol Cell Pharmacol*. 2011; 3:83–92. [PubMed: 22468167]
24. Rodriguez A, Vigorito E, Clare S, Warren MV, Couttet P, Soond DR, van Dongen S, Grocock RJ, Das PP, Miska EA, Vetrie D, Okkenhaug K, Enright AJ, Dougan G, Turner M, Bradley A. Requirement of bic/microRNA-155 for normal immune function. *Science*. 2007; 316:608–611. [PubMed: 17463290]
25. Chong MM, Rasmussen JP, Rudensky AY, Littman DR. The RNaseIII enzyme Drosha is critical in T cells for preventing lethal inflammatory disease. *J Exp Med*. 2008; 205:2005–2017. [PubMed: 18725527]
26. Liston A, Lu LF, O'Carroll D, Tarakhovsky A, Rudensky AY. Dicer-dependent microRNA pathway safeguards regulatory T cell function. *J Exp Med*. 2008; 205:1993–2004. [PubMed: 18725526]
27. Rossi RL, Rossetti G, Wenandy L, Curti S, Ripamonti A, Bonnal RJ, Birolo RS, Moro M, Crosti MC, Gruarin P, Maglie S, Marabita F, Mascheroni D, Parente V, Comelli M, Trabucchi E, De Francesco R, Geginat J, Abrignani S, Pagani M. Distinct microRNA signatures in human lymphocyte subsets and enforcement of the naive state in CD4⁺ T cells by the microRNA miR-125b. *Nat Immunol*. 2011; 12:796–803. [PubMed: 21706005]
28. Ma F, Xu S, Liu X, Zhang Q, Xu X, Liu M, Hua M, Li N, Yao H, Cao X. The microRNA miR-29 controls innate and adaptive immune responses to intracellular bacterial infection by targeting interferon-gamma. *Nat Immunol*. 2011; 12:861–869. [PubMed: 21785411]
29. Derrick T, Roberts CH, Rajasekhar M, Burr SE, Joof H, Makalo P, Bailey RL, Mabey DC, Burton MJ, Holland MJ. Conjunctival MicroRNA Expression in Inflammatory Trachomatous Scarring. *PLoS Negl Trop Dis*. 2013; 7:e2117. [PubMed: 23516655]
30. Igietseme JU, Omosun Y, Partin J, Goldstein J, He Q, Joseph K, Ellerson D, Ansari U, Eko FO, Bandea C, Zhong G, Black CM. Prevention of *Chlamydia*-induced infertility by inhibition of local caspase activity. *J Infect Dis*. 2013; 207:1095–1104. [PubMed: 23303804]
31. Kelly KA, Walker JC, Jameel SH, Gray HL, Rank RG. Differential regulation of CD4 lymphocyte recruitment between the upper and lower regions of the genital tract during *Chlamydia trachomatis* infection. *Infect Immun*. 2000; 68:1519–1528. [PubMed: 10678969]

32. Marks E, Tam MA, Lycke NY. The female lower genital tract is a privileged compartment with IL-10 producing dendritic cells and poor Th1 immunity following *Chlamydia trachomatis* infection. PLoS Pathog. 2010; 6:e1001179. [PubMed: 21079691]
33. Murthy AK, Chambers JP, Meier PA, Zhong G, Arulanandam BP. Intranasal vaccination with a secreted chlamydial protein enhances resolution of genital *Chlamydia muridarum* infection, protects against oviduct pathology, and is highly dependent upon endogenous gamma interferon production. Infect Immun. 2007; 75:666–676. [PubMed: 17118987]
34. Cong Y, Jupelli M, Guentzel MN, Zhong G, Murthy AK, Arulanandam BP. Intranasal immunization with chlamydial protease-like activity factor and CpG deoxynucleotides enhances protective immunity against genital *Chlamydia muridarum* infection. Vaccine. 2007; 25:3773–3780. [PubMed: 17349723]
35. Li W, Guentzel MN, Seshu J, Zhong G, Murthy AK, Arulanandam BP. Induction of cross-serovar protection against genital chlamydial infection by a targeted multisubunit vaccination approach. Clin Vaccine Immunol. 2007; 14:1537–1544. [PubMed: 17942608]
36. Murthy AK, Li W, Guentzel MN, Zhong G, Arulanandam BP. Vaccination with the defined chlamydial secreted protein CPAF induces robust protection against female infertility following repeated genital chlamydial challenge. Vaccine. 2011; 29:2519–2522. [PubMed: 21300093]
37. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{−(Delta Delta C(T))} Method. Methods. 2001; 25:402–408. [PubMed: 11846609]
38. Raphael I, Mahesula S, Kalsaria K, Kotagiri V, Purkar AB, Anjanappa M, Shah D, Pericherla V, Jadhav YL, Raghunathan R, Vaynberg M, Noriega D, Grimaldo NH, Wenk C, Gelfond JA, Forsthuber TG, Haskins WE. Microwave and magnetic (M(2)) proteomics of the experimental autoimmune encephalomyelitis animal model of multiple sclerosis. Electrophoresis. 2012; 33:3810–3819. [PubMed: 23161666]
39. Thibault PA, Huys A, Dhillon P, Wilson JA. MicroRNA-122-dependent and -independent replication of Hepatitis C Virus in Hep3B human hepatoma cells. Virology. 2013; 436:179–190. [PubMed: 23245472]
40. Lu M, Zhang Q, Deng M, Miao J, Guo Y, Gao W, Cui Q. An analysis of human microRNA and disease associations. PLoS One. 2008; 3:e3420. [PubMed: 18923704]
41. Carey AJ, Cunningham KA, Hafner LM, Timms P, Beagley KW. Effects of inoculating dose on the kinetics of *Chlamydia muridarum* genital infection in female mice. Immunol Cell Biol. 2009; 87:337–343. [PubMed: 19204735]
42. Maxion HK, Liu W, Chang MH, Kelly KA. The infecting dose of *Chlamydia muridarum* modulates the innate immune response and ascending infection. Infect Immun. 2004; 72:6330–6340. [PubMed: 15501762]
43. Chaudhuri AA, So AY, Sinha N, Gibson WS, Taganov KD, O'Connell RM, Baltimore D. MicroRNA-125b potentiates macrophage activation. J Immunol. 2011; 187:5062–5068. [PubMed: 22003200]
44. Liu S, Guo W, Shi J, Li N, Yu X, Xue J, Fu X, Chu K, Lu C, Zhao J, Xie D, Wu M, Cheng S, Liu S. MicroRNA-135a contributes to the development of portal vein tumor thrombus by promoting metastasis in hepatocellular carcinoma. J Hepatol. 2012; 56:389–396. [PubMed: 21888875]
45. Li W, Murthy AK, Guentzel MN, Seshu J, Forsthuber TG, Zhong G, Arulanandam BP. Antigen-specific CD4⁺ T cells produce sufficient IFN-gamma to mediate robust protective immunity against genital *Chlamydia muridarum* infection. J Immunol. 2008; 180:3375–3382. [PubMed: 18292563]
46. Igietseme JU, Magee DM, Williams DM, Rank RG. Role for CD8⁺ T cells in antichlamydial immunity defined by *Chlamydia*-specific T-lymphocyte clones. Infect Immun. 1994; 62:5195–5197. [PubMed: 7927806]
47. Roan NR, Starnbach MN. Antigen-specific CD8⁺ T cells respond to *Chlamydia trachomatis* in the genital mucosa. J Immunol. 2006; 177:7974–7979. [PubMed: 17114470]
48. Garg M, Potter JA, Abrahams VM. Identification of microRNAs that regulate TLR2-mediated trophoblast apoptosis and inhibition of IL-6 mRNA. PLoS One. 2013; 8:e77249. [PubMed: 24143215]

49. Hamby M, Coppola G, Ao Y, Geschwind D, Khakh B, Sofroniew M. Inflammatory mediators alter the astrocyte transcriptome and calcium signaling elicited by multiple g-protein-coupled receptors. *J Neurosci.* 2012; 32:14489–14510. [PubMed: 23077035]
50. Yuan H, Niu Y, Liu X, Yang F, Niu W, Fu L. Proteomic analysis of skeletal muscle in insulin-resistant mice: response to 6-week aerobic exercise. *PLoS One.* 2013; 8:e53887. [PubMed: 23326526]
51. Menzies BE. The role of fibronectin binding proteins in the pathogenesis of *Staphylococcus aureus* infections. *Curr Opin Infect Dis.* 2003; 16:225–229. [PubMed: 12821812]
52. Schwarz-Linek U, Werner JM, Pickford AR, Gurusiddappa S, Kim JH, Pilka ES, Briggs JA, Gough TS, Hook M, Campbell ID, Potts JR. Pathogenic bacteria attach to human fibronectin through a tandem beta-zipper. *Nature.* 2003; 423:177–181. [PubMed: 12736686]
53. Goo SY, Lee HJ, Kim WH, Han KL, Park DK, Lee HJ, Kim SM, Kim KS, Lee KH, Park SJ. Identification of OmpU of *Vibrio vulnificus* as a fibronectin-binding protein and its role in bacterial pathogenesis. *Infect Immun.* 2006; 74:5586–5594. [PubMed: 16988233]
54. Kleba BJ, Banta E, Lindquist EA, Stephens RS. Recruitment of mammalian cell fibronectin to the surface of *Chlamydia trachomatis*. *Infect Immun.* 2002; 70:3935–3938. [PubMed: 12065538]
55. Sugars RV, Waddington RJ, Embery G. The interaction of recombinant decorin with alpha2HS-glycoprotein-implications for structural and functional investigations. *Protein Expr Purif.* 2002; 25:180–188. [PubMed: 12071714]
56. Schmidtchen A, Frick IM, Bjorck L. Dermatan sulphate is released by proteinases of common pathogenic bacteria and inactivates antibacterial alpha-defensin. *Mol Microbiol.* 2001; 39:708–713. [PubMed: 11169110]
57. Brown EL, Wooten RM, Johnson BJ, Iozzo RV, Smith A, Dolan MC, Guo BP, Weis JJ, Hook M. Resistance to Lyme disease in decorin-deficient mice. *J Clin Invest.* 2001; 107:845–852. [PubMed: 11285303]
58. Molleken K, Becker E, Hegemann JH. The *Chlamydia pneumoniae* invasin protein Pmp21 recruits the EGF receptor for host cell entry. *PLoS Pathog.* 2013; 9:e1003325. [PubMed: 23633955]
59. Santra M, Reed CC, Iozzo RV. Decorin binds to a narrow region of the epidermal growth factor (EGF) receptor, partially overlapping but distinct from the EGF-binding epitope. *J Biol Chem.* 2002; 277:35671–35681. [PubMed: 12105206]
60. Rothfuchs AG, Trumstedt C, Mattei F, Schiavoni G, Hidmark A, Wigzell H, Rottenberg ME. STAT1 regulates IFN-alpha beta- and IFN-gamma-dependent control of infection with *Chlamydia pneumoniae* by nonhemopoietic cells. *J Immunol.* 2006; 176:6982–6990. [PubMed: 16709859]
61. Lad SP, Fukuda EY, Li J, de la Maza LM, Li E. Up-regulation of the JAK/STAT1 signal pathway during *Chlamydia trachomatis* infection. *J Immunol.* 2005; 174:7186–7193. [PubMed: 15905563]
62. Christian J, Vier J, Paschen SA, Hacker G. Cleavage of the NF-kappaB family protein p65/RelA by the chlamydial protease-like activity factor (CPAF) impairs proinflammatory signaling in cells infected with *Chlamydiae*. *J Biol Chem.* 2010; 285:41320–41327. [PubMed: 21041296]
63. Rodel J, Groh A, Vogelsang H, Lehmann M, Hartmann M, Straube E. Beta interferon is produced by *Chlamydia trachomatis*-infected fibroblast-like synoviocytes and inhibits gamma interferon-induced HLA-DR expression. *Infect Immun.* 1998; 66:4491–4495. [PubMed: 9712805]
64. Zhong G, Fan T, Liu L. *Chlamydia* inhibits interferon gamma-inducible major histocompatibility complex class II expression by degradation of upstream stimulatory factor 1. *J Exp Med.* 1999; 189:1931–1938. [PubMed: 10377188]
65. de la Torre E, Mulla MJ, Yu AG, Lee SJ, Kavathas PB, Abrahams VM. Chlamydia trachomatis infection modulates trophoblast cytokine/chemokine production. *J Immunol.* 2009; 182:3735–3745. [PubMed: 19265152]
66. Kim SW, Ramasamy K, Bouamar H, Lin AP, Jiang D, Aguiar RC. MicroRNAs miR-125a and miR-125b constitutively activate the NF-kappaB pathway by targeting the tumor necrosis factor alpha-induced protein 3 (TNFAIP3, A20). *Proc Natl Acad Sci U S A.* 2012; 109:7865–7870. [PubMed: 22550173]
67. Tan G, Niu J, Shi Y, Ouyang H, Wu ZH. NF-kappaB-dependent microRNA-125b up-regulation promotes cell survival by targeting p38alpha upon ultraviolet radiation. *J Biol Chem.* 2012; 287:33036–33047. [PubMed: 22854965]

68. Wang Z, Filgueiras LR, Wang S, Serezani AP, Peters-Golden M, Jancar S, Serezani CH. Leukotriene B4 Enhances the Generation of Proinflammatory MicroRNAs To Promote MyD88-Dependent Macrophage Activation. *J Immunol.* 2014; 192:2349–2356. [PubMed: 24477912]
69. Warner N, Nunez G. MyD88: a critical adaptor protein in innate immunity signal transduction. *J Immunol.* 2013; 190:3–4. [PubMed: 23264668]
70. Xu S, Witmer PD, Lumayag S, Kovacs B, Valle D. MicroRNA (miRNA) transcriptome of mouse retina and identification of a sensory organ-specific miRNA cluster. *J Biol Chem.* 2007; 282:25053–25066. [PubMed: 17597072]
71. Song L, Liu L, Wu Z, Li Y, Ying Z, Lin C, Wu J, Hu B, Cheng SY, Li M, Li J. TGF-beta induces miR-182 to sustain NF-kappaB activation in glioma subsets. *J Clin Invest.* 2012; 122:3563–3578. [PubMed: 23006329]
72. Stittrich AB, Haftmann C, Sgouroudis E, Kuhl AA, Hegazy AN, Panse I, Riedel R, Flossdorf M, Dong J, Fuhrmann F, Heinz GA, Fang Z, Li N, Bissels U, Hatam F, Jahn A, Hammoud B, Matz M, Schulze FM, Baumgrass R, Bosio A, Mollenkopf HJ, Grun J, Thiel A, Chen W, Hofer T, Loddenkemper C, Lohning M, Chang HD, Rajewsky N, Radbruch A, Mashregi MF. The microRNA miR-182 is induced by IL-2 and promotes clonal expansion of activated helper T lymphocytes. *Nat Immunol.* 2010; 11:1057–1062. [PubMed: 20935646]
73. Mogensen TH. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin Microbiol Rev.* 2009; 22:240–273. [PubMed: 19366914]
74. Bezradica JS, Medzhitov R. Integration of cytokine and heterologous receptor signaling pathways. *Nat Immunol.* 2009; 10:333–339. [PubMed: 19295629]
75. Pedersen IM, Cheng G, Wieland S, Volinia S, Croce CM, Chisari FV, David M. Interferon modulation of cellular microRNAs as an antiviral mechanism. *Nature.* 2007; 449:919–922. [PubMed: 17943132]
76. Frazer LC, Sullivan JE, Zurenski MA, Mintus M, Tomasak TE, Prantner D, Nagarajan UM, Darville T. CD4⁺ T cell expression of MyD88 is essential for normal resolution of *Chlamydia muridarum* genital tract infection. *J Immunol.* 2013; 191:4269–4279. [PubMed: 24038087]
77. Kampinga HH, Garrido C. HSPBs: small proteins with big implications in human disease. *Int J Biochem Cell Biol.* 2012; 44:1706–1710. [PubMed: 22721753]
78. Nagaraja GM, Kaur P, Neumann W, Asea EE, Bausero MA, Multhoff G, Asea A. Silencing Hsp25/Hsp27 gene expression augments proteasome activity and increases CD8⁺ T-cell-mediated tumor killing and memory responses. *Cancer Prev Res (Phila).* 2012; 5:122–137. [PubMed: 22185976]
79. Wang Y, Xiong X, Wu D, Wang X, Wen B. Efficient activation of T cells by human monocyte-derived dendritic cells (HMDCs) pulsed with *Coxiella burnetii* outer membrane protein Com1 but not by HspB-pulsed HMDCs. *BMC Immunol.* 2011; 12:52. [PubMed: 21888659]
80. Gangneux C, Daveau M, Hiron M, Derambure C, Papaconstantinou J, Salier JP. The inflammation-induced down-regulation of plasma Fetuin-A (alpha2HS-Glycoprotein) in liver results from the loss of interaction between long C/EBP isoforms at two neighbouring binding sites. *Nucleic Acids Res.* 2003; 31:5957–5970. [PubMed: 14530444]
81. Lei L, Li Z, Zhong G. Rottlerin-mediated inhibition of *Chlamydia trachomatis* growth and uptake of sphingolipids is independent of p38-regulated/activated protein kinase (PRAK). *PLoS One.* 2012; 7:e44733. [PubMed: 22970301]
82. Mathur SP, Holt VL, Lee JH, Jiang H, Rust PF. Levels of antibodies to transferrin and alpha 2-HS glycoprotein in women with and without endometriosis. *Am J Reprod Immunol.* 1998; 40:69–73. [PubMed: 9764347]
83. Mathur SP, Lee JH, Jiang H, Arnaud P, Rust PF. Levels of transferrin and alpha 2-HS glycoprotein in women with and without endometriosis. *Autoimmunity.* 1999; 29:121–127. [PubMed: 10433073]
84. Petracco R, Grechukhina O, Popkhadze S, Massasa E, Zhou Y, Taylor HS. MicroRNA 135 regulates HOXA10 expression in endometriosis. *J Clin Endocrinol Metab.* 2011; 96:E1925–E1933. [PubMed: 21956427]

85. Lee TS, Jeon HW, Kim YB, Kim YA, Kim MA, Kang SB. Aberrant microRNA expression in endometrial carcinoma using formalin-fixed paraffin-embedded (FFPE) tissues. PLoS One. 2013; 8:e81421. [PubMed: 24363810]
86. Kim RY, JCH MR, Starkey AT, Eessilfie PS, Foster PM. Hansbro: MicroRNA Inhibition in Neonatal *Chlamydia* Lung Infection prevents infection-induced lung pathology in later life. Respirology. 2013; 18:38–85.
87. Jupelli M, Murthy AK, Chaganty BK, Guentzel MN, Selby DM, Vasquez MM, Mustafa SB, Henson BM, Seidner SR, Zhong G, Arulanandam BP. Neonatal chlamydial pneumonia induces altered respiratory structure and function lasting into adult life. Lab Invest. 2011; 91:1530–1539. [PubMed: 21769086]
88. Richard, YK.; Jay, CH.; Malcolm, RS.; Ama Tawiah, E.; Paul, SF.; Philip, MH. MicroRNAs In Lung Health And Disease. American Thoracic Society; 2013. Inhibition Of Early-Life *Chlamydia* Lung Infection-Induced MicroRNAs Prevents Infection-Induced Lung Pathologies In Later Life; p. A3418-A3418.
89. Wang W, Stassen FR, Surcel HM, Ohman H, Tiitinen A, Paavonen J, de Vries HJ, Heijmans R, Pleijster J, Morre SA, Ouburg S. Analyses of polymorphisms in the inflammasome-associated NLRP3 and miRNA-146A genes in the susceptibility to and tubal pathology of *Chlamydia trachomatis* infection. Drugs Today (Barc). 2009; 45(Suppl B):95–103. [PubMed: 20011700]

Fig. 1. *C. 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 RT² 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/timepoint).

Fig. 2. Bacterial Burden in Mock Vaccinated and rCPAF Vaccinated Mice Following *C. muridarum* Infection

Resolution of *C. muridarum* genital infection in mock vaccinated *C. mur* challenged (Black Bars) and rCPAF vaccinated *C. mur* challenged (White 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 $\pm$ SD 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.

Fig. 3. Increasing Dose *C. muridarum* Modulate Infection Associated miRs Expression

Increased inoculating dose of *C. muridarum* differentially modulate miRs but not significantly (A) Fold changes in miR expression were calculated based on differences in *C. muridarum* 5×10^5 or 5×10^6 IFU infected and 5×10^4 IFU infected mice. Fold changes in miR expression were calculated based on differences and plotted as ratio of miR expression in 5×10^4 *C. mur*/Mock infected (Black bars); or 5×10^5 *C. mur*/Mock infected (Grey bars) or 5×10^6 *C. mur*/Mock infected (White bars). All miR expression analyses were normalized to RNU6 or SNORD68 expression values and calculated using RT² 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 5×10^4 *C. mur*/Mock infected; 5×10^5 *C. mur*/Mock infected; 5×10^6 *C. mur*/Mock infected; # $P < 0.05$ intra-group comparison between black, grey and white bars; ◆ ANOVA with Tukey B correction

Fig. 4. *C. 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 h 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- κ B(6 h post infection), STAT-1 or IRF-1 (24 h post infection). Activation of inflammatory pathways was calculated as the difference in luciferase activity between *C. muridarum* infected wells (TF) and *C. muridarum* infected wells co-transfected with miR inhibitors. 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. * $P<0.05$.

Fig. 5. Host Immune Response Against *C. 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.

C57BL/6 (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). (B) 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. * $P < 0.05$ HSPB1, Heat shock protein B1; AHSG, Alpha-2HS-Glycoprotein.

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Fig. 7. *C. 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.

C. muridarum Genital Infection Modulates Host microRNA Responses in a Spatio-Temporal Fashion**TABLE 1**

<i>C. muridarum</i> Infection									
Day 6					Day 12				
miR	Lower GT Fold Change	<i>p</i> value	Upper GT Fold Change	<i>p</i> value	Lower GT Fold Change	<i>p</i> value	Upper GT Fold Change	<i>p</i> 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	