UC Merced

UC Merced Previously Published Works

Title

MicroRNAs Modulate Pathogenesis Resulting from Chlamydial Infection in Mice

Permalink

https://escholarship.org/uc/item/3qh2285j

Journal

Infection and Immunity, 85(1)

ISSN

0019-9567

Authors

Yeruva, Laxmi
Pouncey, Dakota L
Eledge, Michael R
et al.

Publication Date

2017

DOI

10.1128/iai.00768-16

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

AMERICAN SOCIETY FOR MICROBIOLOGY | Infection and Immunity®

HOST RESPONSE AND INFLAMMATION

MicroRNAs Modulate Pathogenesis Resulting from Chlamydial Infection in Mice

Laxmi Yeruva,[a,b,c] Dakota L. Pouncey,[a,b,c] Michael R. Eledge,[a,b,c] Sudeepa Bhattacharya,[a,b,c] Chunqiao Luo,[a,c] Erin W. Weatherford,[a,b] David M. Ojcius,[d] Roger G. Rank[a,b,c]

Department of Pediatrics, University of Arkansas for Medical Sciences,[a] Arkansas Children's Research Institute,[b] and Arkansas Children's Nutrition Center,[c] Little Rock, Arkansas, USA; Department of Biomedical Sciences, University of the Pacific, Arthur Dugoni School of Dentistry, San Francisco, California, USA[d]

ABSTRACT Not all women infected with chlamydiae develop upper genital tract disease, but the reason(s) for this remains undefined. Host genetics and hormonal changes associated with the menstrual cycle are possible explanations for variable infection outcomes. It is also possible that disease severity depends on the virulence of the chlamydial inoculum. It is likely that the inoculum contains multiple genetic variants, differing in virulence. If the virulent variants dominate, then the individual is more likely to develop severe disease. Based on our previous studies, we hypothesized that the relative degree of virulence of a chlamydial population dictates the microRNA (miRNA) expression profile of the host, which, in turn, through regulation of the host inflammatory response, determines disease severity. Thus, we infected C57BL/6 mice with two populations of *Chlamydia muridarum*, each comprised of multiple genetic variants and differing in virulence: an attenuated strain ($Nigg_A$) and a virulent strain ($Nigg_V$). $Nigg_A$ and $Nigg_V$ elicited upper tract pathology in 54% and 91% of mice, respectively. miRNA expression analysis in $Nigg_V$-infected mice showed significant downregulation of miRNAs involved in dampening fibrosis (miR-200b, miR-200b-5p, and 200b-3p miR-200a-3p) and in transcriptional regulation of cytokine responses (miR-148a-3p, miR-152-3p, miR-132, and miR-212) and upregulation of profibrotic miRNAs (miR-142, and miR-147). Downregulated miRNAs were associated with increased expression of interleukin 8 (IL-8), CXCL2, IL-1β, tumor necrosis factor alpha (TNF-α), and IL-6. Infection with $Nigg_V$ but not $Nigg_A$ led to decreased expression of Dicer and Ago 2, suggesting that $Nigg_V$ interaction with host cells inhibits expression of the miRNA biogenesis machinery, leading to increased cytokine expression and pathology.

KEYWORDS *Chlamydia*, miRNA, Dicer, Ago2

Chlamydia trachomatis is the most common bacterial sexually transmitted infection in the world, and the majority of infections occur in young adults and adolescents, often leading to fibrosis, tubal obstruction, infertility, chronic pelvic pain, and pelvic inflammatory disease (PID) (1, 2). It is not known why some individuals infected with *Chlamydia* develop severe upper tract disease while others do not. A number of factors may be associated with varied outcomes, including host genetic background, vaginal microbiota, or hormonal contraception, but disease severity may also be determined by the particular genetic chlamydial variant(s) comprising the inoculum. Recently, we have characterized two distinct clonal chlamydial variants. The virulent variant elicited upper tract pathology in the majority of animals and induced increased expression of various inflammatory cytokines, while the attenuated variant resulted in minimal pathology and low expression of inflammatory cytokines (3). Interestingly, we found that the

Received 7 September 2016 **Returned for modification** 10 October 2016 **Accepted** 26 October 2016

Accepted manuscript posted online 31 October 2016

Citation Yeruva L, Pouncey DL, Eledge MR, Bhattacharya S, Luo C, Weatherford EW, Ojcius DM, Rank RG. 2017. MicroRNAs modulate pathogenesis resulting from chlamydial infection in mice. Infect Immun 85:e00768-16. https://doi.org/10.1128/IAI.00768-16.

Editor Craig R. Roy, Yale University School of Medicine

Copyright © 2016 American Society for Microbiology. All Rights Reserved.

Address correspondence to Laxmi Yeruva, vlyeruva@uams.edu.

profile of microRNAs (miRNAs) elicited in the host with each variant infection was profoundly different as a function of the variant, with a lower expression of 6 miRNAs observed during infection with the virulent variant and higher expression with the attenuated variant. These data suggest that miRNAs expressed by the interaction between *Chlamydia* and host may regulate the inflammatory process and level of pathology.

MicroRNAs are abundant, evolutionarily conserved noncoding small RNAs (~22 nucleotides) that are transcribed in the nucleus as long polyadenylated pri-miRNAs. Drosha, an endonuclease, and cofactor DiGeorge syndrome critical region 8 (DGCR8) process the pri-miRNA to yield pre-miRNA hairpin structures of 60 to 100 bp (4). Exportin 5 transports the pre-miRNA into the cytoplasm, and pre-miRNA is processed in a canonical or noncanonical pathway to release mature miRNA. In canonical pathways, both Dicer and Ago2 are involved in processing the pre-miRNA to release the mature miRNA. However, in the noncanonical pathway, mature miRNA is released by Ago2 and eIF1A complex in a Dicer-independent fashion (5–7). These mature miRNAs are involved in regulating multiple physiological and pathological processes (8, 9). It has been estimated that miRNAs target 20% to 30% of human mRNAs that affect transcription, transduction, growth, and fatty acid metabolism. miRNAs have been shown to be immune modulators serving as an important link between innate and adaptive immune responses, and dysregulation of miRNA expression has been linked to a number of diseases, such as cardiovascular disease, cancer, and infectious and metabolic diseases (8–10). In addition, previous reports have indicated that downregulation of Dicer impacts expression of only a few dozen miRNAs, and furthermore, only minor alterations were observed in the miRNA profile in the oviducts and uteri of Dicer conditional knockout mice (11–15). All these data suggest that a large number of miRNAs may be produced in the female reproductive tract through the noncanonical pathway (Ago2). Furthermore, it has been shown that the abundance of miRNAs dictates the stability of Dicer and Ago2, and in turn, Ago proteins regulate miRNA stability (16, 17). For example, when several miRNAs were downregulated, Ago2 protein was degraded (16). All of these reports suggest that expression of miRNAs is regulated by the biogenesis machinery, and every protein in the biogenesis pathway could have an impact on expression of specific miRNAs.

miRNAs are involved in regulating inflammatory signaling pathways during viral and bacterial infections, transplantation rejection, and autoimmunity (18–23). For example, a differential expression profile of circulating (serum) miRNA has been observed during hepatitis C virus infection (24, 25) and during *Helicobacter*, *Mycobacterium*, *Salmonella*, and *Listeria* infections (9, 26, 27). In addition, differential miRNA expression was associated with inflammatory scarring in patients with ocular *Chlamydia trachomatis* infection (28). Furthermore, during *C. trachomatis* serovar L2 infection in a mouse model, miRNAs were shown to be involved in controlling epithelial mesenchymal transition (EMT), fibrosis, and tumorigenesis. These data suggest that expression of miRNAs is altered during the inflammatory process and fibrosis. However, until now, there have been no studies demonstrating that early miRNA expression could be associated with inflammatory responses and chronic pathology following infection with a mixture of microbial variants.

It has usually been assumed that chlamydial infection of an individual consists of a single chlamydial strain that is passed from person to person with little variance. However, it has become clear that a person is in fact infected with a population of multiple genetic variants. Initially, Miyairi and colleagues recognized that there are pathogenic differences among serovars of *C. trachomatis*. They reported that ocular serovars had longer developmental cycles and lower growth rates than genital serovars and related this difference to their pathotypes (29). Along the same line, Kari et al. tested two different isolates of serovar A and observed that one isolate, which had smaller plaques, slower growth, and increased sensitivity to gamma interferon (IFN-γ), resulted in a shorter infection in nonhuman primates, with a lower number of inclusion-forming units (IFU), and produced less pathology during conjunctival infection (30).

Sturdevant and colleagues showed that both virulent and avirulent variants were present in the same population of *C. trachomatis* serovar D, and the variants accounted for differences among mice in the length of infection (31). Thus, it is clear that even within a given serovar, significant variation in virulence can exist. Finally, we observed different size plaques in clinical samples obtained from patients infected with chlamydiae (L. Yeruva and B. Batteiger, unpublished data). The samples were only one passage away from the actual patient swab, further suggesting that patients are infected with multiple variants. Thus, the data suggest that patients are likely infected with a mixture of variants and that the relative pathogenicity of the population of variants determines the severity and outcome of infection.

Thus, in this study, we utilized two distinct populations of *C. muridarum*, one having an attenuated phenotype ($Nigg_A$) and the other a virulent phenotype ($Nigg_V$). $Nigg_A$ is the Nigg strain of *C. muridarum* that has been sequentially passaged in our laboratory since it was obtained as a yolk sac preparation in 1977. Recently, Jasper and colleagues (32) genetically characterized 30 plaques from this strain, originally obtained from our laboratory, and found a great variety of genotypes, indicating that multiple genetic variants are present in $Nigg_A$ and that it is indeed a mixed population. In a more limited study, we isolated 4 distinct genetic variants from the $Nigg_A$ strain (3). $Nigg_V$ was a passage of $Nigg_A$ which spontaneously demonstrated a virulent phenotype, suggesting that an increase in the representation of virulent variants had occurred during passage. The exact percentage representation of the variants in both $Nigg_A$ and $Nigg_V$ has not been determined, although Jasper et al. have presented a summary of important single nucleotide polymorphisms (SNPs) for $Nigg_A$ (32). Chen and colleagues have shown that a change in variant representation in *C. muridarum* can occur through *in vitro* passage (33). Therefore, we used these two populations to determine if disease outcome during infection with a population of variants is associated with expression of a given miRNA profile. We further investigated whether expression of the miRNA biogenesis machinery is impacted during infection with attenuated and virulent populations of chlamydiae.

RESULTS

***In vivo* growth phenotype and pathogenicity of $Nigg_A$ and $Nigg_V$ in the genital infection model.** In this study, we utilized two distinct populations of the Nigg strain of *C. muridarum*, one being relatively attenuated ($Nigg_A$) and the other being more virulent ($Nigg_V$). Initially, we characterized the course of genital infection in mice with each population. Thus, C57BL/6 mice were infected genitally with 3×10^5 IFU and the course of the infection was monitored by measuring the number of IFU in genital tract swabs. Mice infected with $Nigg_V$ had a significantly higher bacterial load than mice infected with $Nigg_A$ (2-factor [days, group] analysis of variance [ANOVA] with repeated measures; $P < 0.0001$), and a longer infection was observed (Fig. 1A). All mice infected with $Nigg_V$ cleared infection between days 27 and 30, while mice infected with $Nigg_A$ cleared infection between days 21 and 24, demonstrating that infection with $Nigg_V$ lasted at least 4 to 5 days longer. Furthermore, when data were adjusted to multiple comparisons for different days, a significantly higher bacterial load was seen on day 3 ($P < 0.0001$) and day 6 ($P < 0.05$) with $Nigg_V$ than with $Nigg_A$ (Fig. 1A).

To determine if $Nigg_V$-infected mice have more pathology than mice infected with $Nigg_A$, animals were euthanized on day 35 of infection and gross pathology in the genital tract was evaluated. The data demonstrated that 54% of the $Nigg_A$-infected mice were positive for hydrosalpinx, while 91% of the $Nigg_V$-infected mice had hydrosalpinx (Fig. 1B). Furthermore, 8 of 22 (36%) oviducts were positive for hydrosalpinx with $Nigg_A$ infection, and 16 of 22 (72%) oviducts were positive for pathology in $Nigg_V$-infected mice. Chi-square analyses with Yates correction determined that there was significantly more pathology in $Nigg_V$-infected mice than in $Nigg_A$-infected mice ($P < 0.05$), confirming that $Nigg_V$ is more pathogenic than $Nigg_A$ (Fig. 1B).

Intranasal infection. To confirm the difference in virulence using a different route of infection, we infected mice by intranasal route as described by Ramsey et al. (34). Prior to infection, initial body weights were measured to show the range in each group

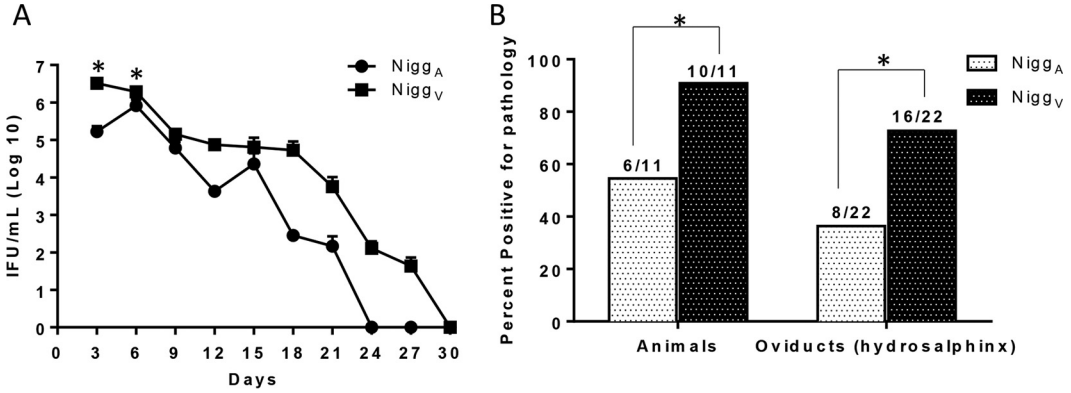

FIG 1 C57BL/6 mice were infected intravaginally with Nigg$_A$ or Nigg$_V$. Data represent averages for 11 animals in each group and are presented as means ± SEM. The experiment was carried out twice with 5 animals per group the first time and 6 animals per group the second time. (A) Swabs were collected from mice infected intravaginally once every 3 days for IFU enumeration to monitor the course of infection. Repeated-measure ANOVA was carried out to determine the significance for the IFU data (*, $P < 0.001$). (B) On day 35, all mice were euthanized for gross pathology observation. The number of mice and oviducts with pathology is presented based on a total of 11 mice and 22 oviducts, respectively. The data were analyzed by Chi-square analyses with Yates correction to determine the significance (*, $P < 0.05$).

(Fig. 2A). The two groups had similar starting body weights. Unpaired *t* test showed no significant difference in body weight between the groups prior to infection (Fig. 2A). Mice were infected with Nigg$_A$ or Nigg$_V$ stocks at 1×10^4 inclusion-forming units (IFU) by the intranasal route, and the change in body weight was monitored daily through the course of infection. Lethality or signs of morbidity were also followed, although they were not noted in any of the mice. Interestingly, both Nigg$_A$- and Nigg$_V$-infected mice lost weight until day 9 but started gaining weight from day 9 onwards (Fig. 2). However, the extent of weight loss was significantly higher in Nigg$_V$-infected mice than in Nigg$_A$-infected mice (2-factor [days, group] analysis of variance with repeated measures; $P < 0.0001$). Furthermore, Nigg$_V$-infected animals had significantly lower body weight from day 5 until day 14 than did Nigg$_A$-infected mice (Fig. 2). We confirmed that animals were indeed infected by measuring the antibody response on day 35 (data not shown).

Expression of cytokines in HeLa cells. Chemokine/cytokine responses have previously been shown to correlate with pathology in chlamydial infections (35–37). It was expected that Nigg$_V$ would induce higher chemokine/cytokine responses than Nigg$_A$,

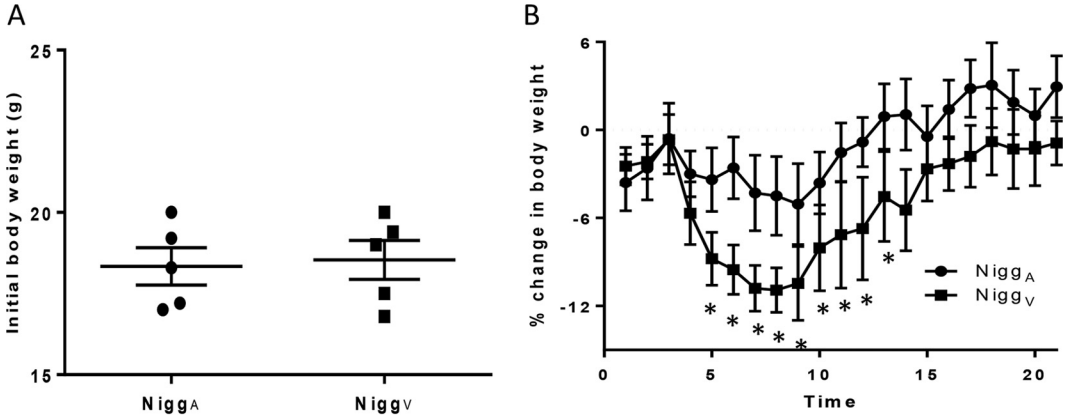

FIG 2 C57BL/6 mice were infected intranasally with Nigg$_A$ or Nigg$_V$. The data represent averages for 5 animals in each group and are presented as means ± SEM. (A) Initial body weights with individual animals are plotted to show the range of weights in each group. An unpaired *t* test between the groups did not show significant differences in body weight prior to infection. (B) Body weights were recorded every day to determine the pathogenicity of Nigg$_A$ or Nigg$_v$ in infected mice. Repeated-measure ANOVA was carried out to determine the significance in Nigg$_V$-infected mice in comparison with Nigg$_A$-infected mice (*, $P < 0.0001$).

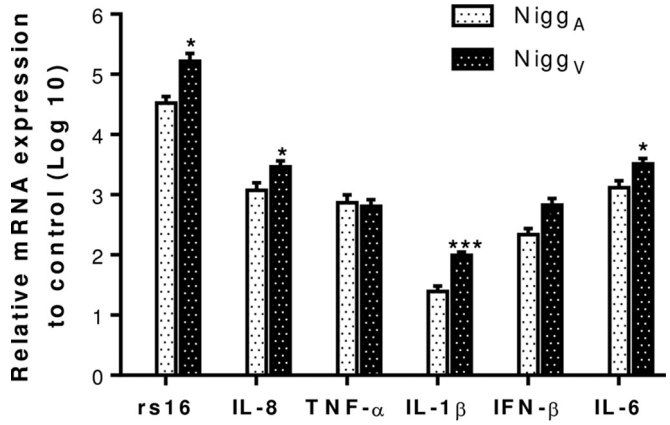

FIG 3 Cytokine expression in human epithelial cells infected with $Nigg_A$ or $Nigg_V$. HeLa cells were infected with $Nigg_A$ or $Nigg_V$, and chemokine/cytokine expression was measured. The data are averages from four independent experiments with 4 mice in each experiment and are presented as means ± SEM. Statistical significance was determined by two-tailed *t* test (*, $P < 0.05$; ***, $P < 0.001$).

as more pathology was observed during $Nigg_V$ infection in mice. Thus, cervical epithelial (HeLa) cells were infected at a multiplicity of infection (MOI) of 1 and were subjected to real-time PCR analyses to measure key proinflammatory molecules that had previously been shown to correlate with pathology during chlamydial infections (interleukin 8 [IL-8], tumor necrosis factor alpha [TNF-α], IL-1β, IFN-β, and IL-6) (36–42). Interestingly, HeLa cells infected with $Nigg_V$ showed significantly higher expression of IL-8, IL-1β, IFN-β, and IL-6, but no difference was observed for TNF-α in comparison with the level induced by $Nigg_A$ (Fig. 3). The significant increase in expression with $Nigg_V$ in comparison with $Nigg_A$ corresponded to 2.4-fold for IL-8, 4-fold for IL-1β, 3-fold for IFN-β, and 2.4-fold for IL-6. We measured *rs*16 to determine chlamydial growth, and significantly higher expression of *rs*16 (5-fold) was observed with $Nigg_V$ infection than with $Nigg_A$ infection in HeLa cells. These data demonstrate that higher $Nigg_V$-induced pathology correlates with higher expression of key proinflammatory molecules.

Expression of cytokines in mice infected with $Nigg_A$ and $Nigg_V$. *In vitro* experiments demonstrated that $Nigg_V$-infected cells have higher expression of cytokines. To determine if the same phenomenon could be observed *in vivo*, C57BL/6 mice were infected with $Nigg_A$ or $Nigg_V$, and CXCL1, CXCL2, TNF-α, IL-1β, IFN-β, and IL-6 were measured in cervical tissue 4 days after infection. The expression of TNF-α, IL-1β, and IL-6 were in fact significantly higher in $Nigg_V$-infected mice than in $Nigg_A$-infected mice (Fig. 4A). The increases in expression during $Nigg_V$ infection were 1.7-fold for CXCL2, 2.4-fold for IL-1β, 1.6-fold for TNF-α, and 4-fold for IL-6. Thus, the *in vivo* data correlate with *in vitro* cytokine measurements observed in human and mouse cells and demonstrate that $Nigg_V$ induces higher inflammatory responses in mice than $Nigg_A$.

Finally, to determine the bacterial load from these mice prior to tissue collection, the day 3 swab was used to enumerate IFU. The data showed significantly higher bacterial burdens in mice infected with the virulent strain, similar to the *in vitro* study ($P < 0.01$ [Fig. 4B]), confirming that higher bacterial infection can lead to increased expression of proinflammatory cytokines *in vivo* and that virulence may be related to the replicative ability of the organism.

Expression of miRNAs in mice infected with $Nigg_A$ and $Nigg_V$. Recently, we have shown that the miRNA expression profile differs depending on the chlamydial variant infecting mice at 24 h after infection in the intracervical model, in which the chlamydiae are injected directly into the endocervix (3). Since infection with the two chlamydial populations results in differing cytokine and chemokine responses, it is likely that a difference in the miRNA expression during each infection exists. Therefore, we used Affymetrix 4.0 chips to assess the miRNA expression profile in mice infected with either the $Nigg_A$ or $Nigg_V$ populations from cervices at 4 days of infection. Microarray data

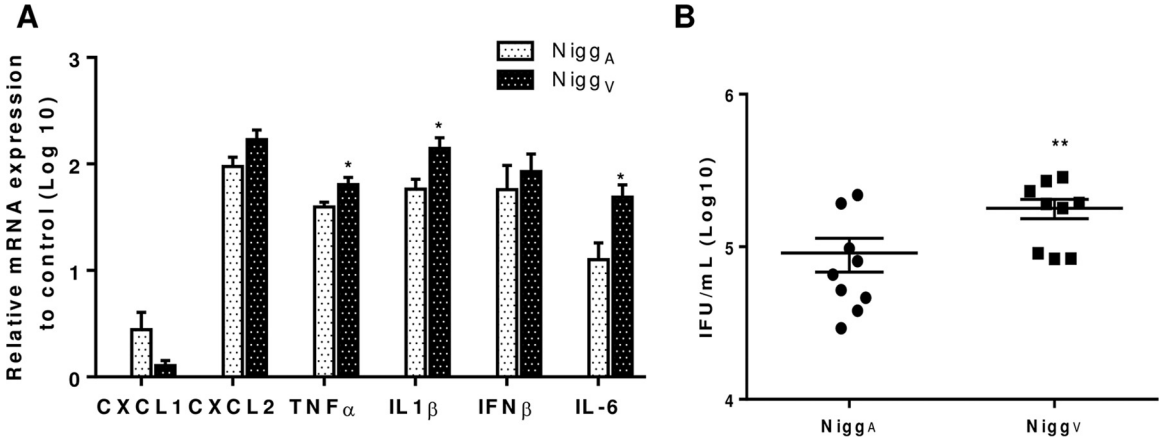

FIG 4 Cytokine expression in cervices and vaginal IFU in mice infected with $Nigg_A$ or $Nigg_V$. (A) Cytokine expression of cervices on day 4 from mice infected with $Nigg_A$ or $Nigg_V$. The data represent values for 9 animals per group and are presented as means ± SEM. Statistical significance was determined by two-tailed *t* test (*, $P < 0.05$). (B) Mice were infected with $Nigg_A$ or $Nigg_V$, and swabs on day 3 of infection were used for IFU determination. The data are from 9 animals/group and are presented as means ± SEM. Statistical significance was determined by Mann-Whitney U test, and a *P* value of <0.05 was considered significant. *, $P < 0.05$; **, $P < 0.01$.

were initially subjected to hierarchical clustering. Data showed 3 of 7 animals infected with $Nigg_A$ being clustered into $Nigg_V$ and 1 of 7 $Nigg_V$-infected animals clustered into $Nigg_A$ in their miRNA expression profile. Furthermore, 2 of 7 $Nigg_A$-infected animals were clustered into controls (see Fig. S1 in the supplemental material). These data showed that few animals infected with $Nigg_A$ develop pathology, as seen in Fig. 1, and suggested that one may be able to predict the extent of pathology based on the miRNA expression pattern (Fig. S1 in the supplemental material).

The data analysis showed that a total of 3,222 miRNAs were expressed and 37 miRNAs were significantly different between the three groups as revealed by the Kruskal-Wallis rank sum test and adjusted by the Benjamini-Hochberg (BH) method ($P < 0.05$). All 37 miRNAs were significantly changed in $Nigg_V$-infected mice, while only 16 miRNAs were significantly different in $Nigg_A$-infected mice in comparison with controls (Table S1 in the supplemental material). Of the 37 miRNAs, 20 were significantly downregulated in $Nigg_V$-infected mice, while only 8 miRNAs were significantly different during $Nigg_A$ infection in comparison with controls. Furthermore, of the 37 miRNAs, 17 were significantly upregulated in $Nigg_V$-infected mice, while 8 were significantly upregulated in $Nigg_A$-infected mice (Table S1 in the supplemental material). We have confirmed the expression of 10 miRNAs by real-time PCR, which showed an expression pattern (Fig. 5B) similar to the microarray data. In $Nigg_V$-infected mice, we observed significant upregulation of miR-142-3p, miR-132, miR-147-3p, miR-212-3p, and miR-149-3p. In contrast, in $Nigg_A$-infected mice, miR-132, miR-147-3p, and miR-212-3p were upregulated relative to values in control mice (Fig. 5B). Furthermore, miR-142-3p, miR-132, and miR-212-3p were expressed at significantly higher levels in $Nigg_V$-infected mice than in $Nigg_A$-infected mice. In addition, in $Nigg_V$-infected mice, miR-200b-5p, miR-200a-3p, miR-411-5p, and miR-100-5p were significantly downregulated, while in $Nigg_A$-infected mice, miR-200b-3p, miR-200b-5p, and miR-200a-3p were downregulated, relative to values in control mice (Fig. 5B).

To determine if differentially expressed miRNAs during chlamydial infection regulate host pathways, canonical pathway analyses were carried out based on the known gene targets of the altered miRNAs. The analyses showed that a total of 82 cellular pathways could potentially be regulated by miRNAs expressed in mice infected with $Nigg_V$ (Fig. S2 in the supplemental material); only the top 20 pathways are shown in Fig. 5C. The highest enrichment score was for pattern recognition receptors (PRRs), dendritic cell maturation, NF-κB signaling, Toll-like receptors (TLRs), interferon, IL-17, IL-6, and death receptor signaling pathways (Fig. 5C). These data indicate that key pathways associated with chlamydial pathogenesis may be differentially regulated by miRNAs depending on the composition of chlamydial variant strains infecting the cervix (35–38, 40, 41).

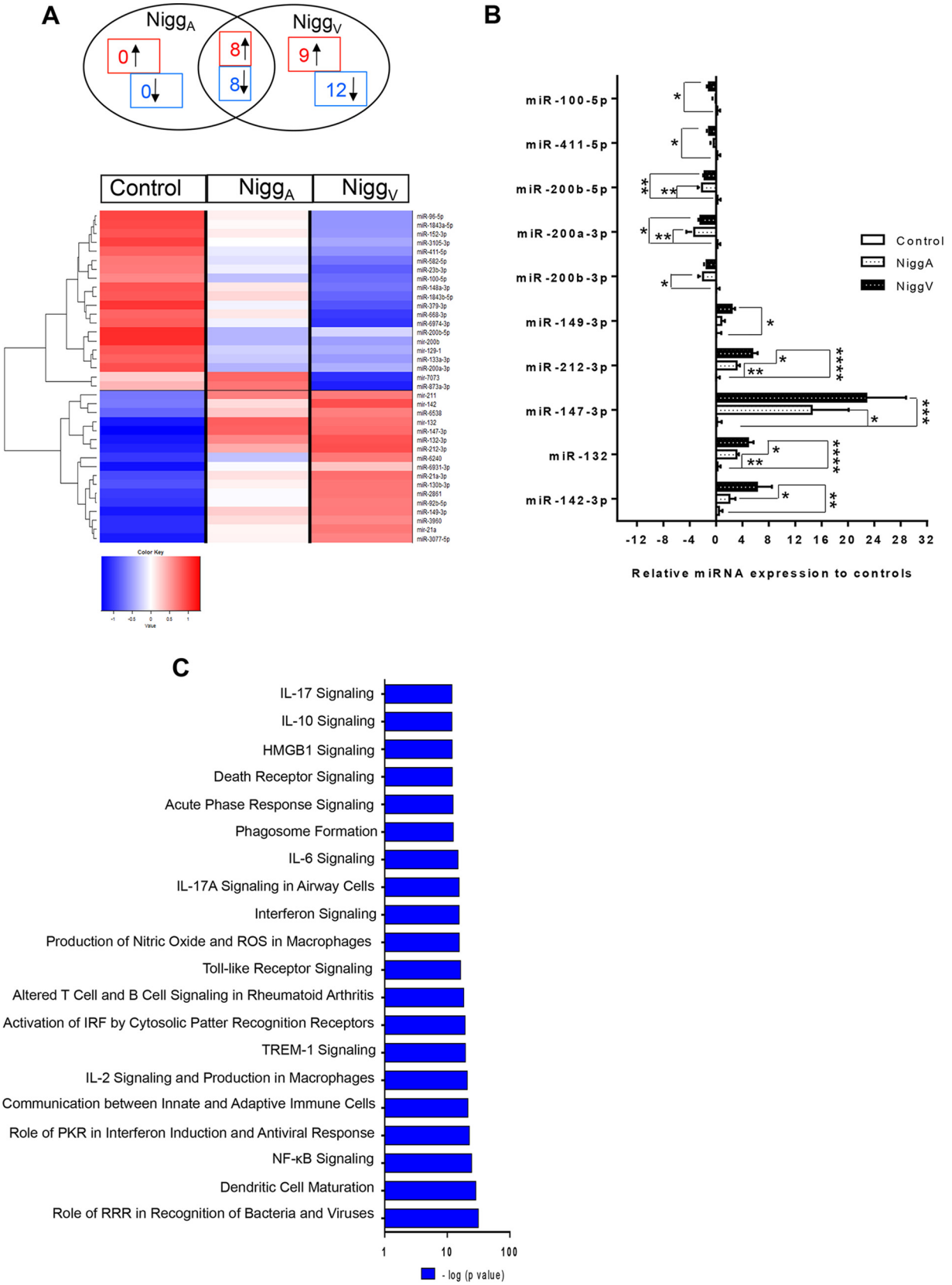

FIG 5 Comparison of differentially expressed miRNAs and pathway analyses in cervices of mice infected with Nigg$_A$ or Nigg$_V$. (A) Venn diagram and heat maps indicate differentially expressed miRNAs in Nigg$_A$- or Nigg$_V$-infected mice relative to those in control mice. Red and blue indicate up- and downregulation, respectively. Fold changes of ≥2.5 or ≤−2.5 are shown. (B) miRNA expression was determined by real-time PCR of RNA samples obtained from mice infected with Nigg$_A$ or Nigg$_V$. The data represent values for 9 animals per group and are presented as means ± SEM.

(Continued on next page)

Expression of the miRNA biogenesis machinery. The miRNA data indicated that $Nigg_V$ infection results in significant downregulation of several miRNAs, suggesting that $Nigg_V$ infection possibly impacts miRNA processing enzymes. Thus, we measured the expression of Dicer and Ago2 proteins by Western blotting in HeLa cells infected with $Nigg_A$ or $Nigg_V$ (Fig. 6A and B). Interestingly, $Nigg_V$ infection altered Dicer and Ago2 expression in comparison with uninfected cells (control); in contrast, $Nigg_A$ infection did not have a significant effect on the expression of both proteins (Fig. 6A and B) in comparison with control. Moreover, $Nigg_V$-infected cells had significantly lower expression of both Dicer and Ago2 than did $Nigg_A$-infected cells (Fig. 6A and B). These data further support our interpretation that $Nigg_V$ infection modifies the expression of the miRNA biogenesis machinery, resulting in significant downregulation of miRNAs during $Nigg_V$ infection. To assess infection from these experiments, supernatants were processed for IFU, and as expected, $Nigg_V$ infection resulted in a higher bacterial load, further suggesting that $Nigg_V$ grows faster.

DISCUSSION

We previously demonstrated that plaque-purified chlamydial variants with vastly contrasting pathological outcomes elicited specific cytokine and miRNA profiles in the host within 24 h of infection (3). However, individuals are most likely infected with a mixed population of genetic variants of *Chlamydia*, and individuals who are infected with populations containing virulent variants are more likely to develop upper tract pathology. Thus, we have used two chlamydial populations in this study ($Nigg_A$ and $Nigg_V$) and extensively characterized the populations in terms of inducing pathology, determined miRNA expression, and measured expression of the miRNA biogenesis machinery during infection with each population. To determine the pathotype in the genital tract model, mice were infected by the vaginal route; on day 35 of infection, significantly more animals and oviducts had pathology among $Nigg_V$-infected mice than among mice infected with $Nigg_A$. Furthermore, $Nigg_V$-infected mice had a higher bacterial burden and longer infection course (Fig. 1). To further determine if the two chlamydial stocks differ in their virulence, C57BL/6 mice were infected by the intranasal route, and their weights were measured. The results showed significant weight loss in $Nigg_V$-infected mice in comparison with $Nigg_A$-infected mice (Fig. 2). All these data indicated that $Nigg_V$ is more virulent in mice than $Nigg_A$. To determine if the early cytokine responses are heightened in $Nigg_V$-infected mice, cytokines previously known to be involved in chlamydial pathology were measured both *in vitro* and *in vivo*. The data demonstrated significantly increased expression of CXCL2, IL-1β, TNF-α, and IL-6 during $Nigg_V$ infection, further indicating that $Nigg_V$ infection induces higher levels of proinflammatory cytokines than $Nigg_A$ (Fig. 3 and 4).

miRNAs have been shown to be differentially expressed during chlamydial infections (3, 28, 43–46). To determine if infection with $Nigg_A$ or $Nigg_V$ has an impact on miRNA expression in mice, cervices infected for 4 days were subjected to microarray analyses. The results showed that miRNAs were differentially expressed and cytokine responses were significantly upregulated in $Nigg_V$-infected mice, correlating with oviduct pathology. A study by Gupta et al. (45) showed significant downregulation of miR-125b-5p, miR-16, miR-214, miR-23b, miR-135a, miR-182, miR-183, miR-30c, and miR-30e on day 6 following genital infection of mice. More recently, it has been demonstrated that during ocular *C. trachomatis* infection, upregulation of miR-155 and downregulation of miR-184 correlated with conjunctival inflammation (43). Furthermore, in our previous study (3) with the attenuated CmVar004 strain and the virulent CmVar001 strain, the miRNA response was measured within the first developmental cycle, whereas in the current study we measured the response on day 4 using an

FIG 5 Legend (Continued)
Statistical significance was determined by one-way ANOVA, and multiple-comparison *post hoc* Tukey test was carried out to compare mean differences between the groups (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.00001$). (C) Canonical-pathway analyses showed 82 different pathways being regulated; only the top 20 pathways are shown.

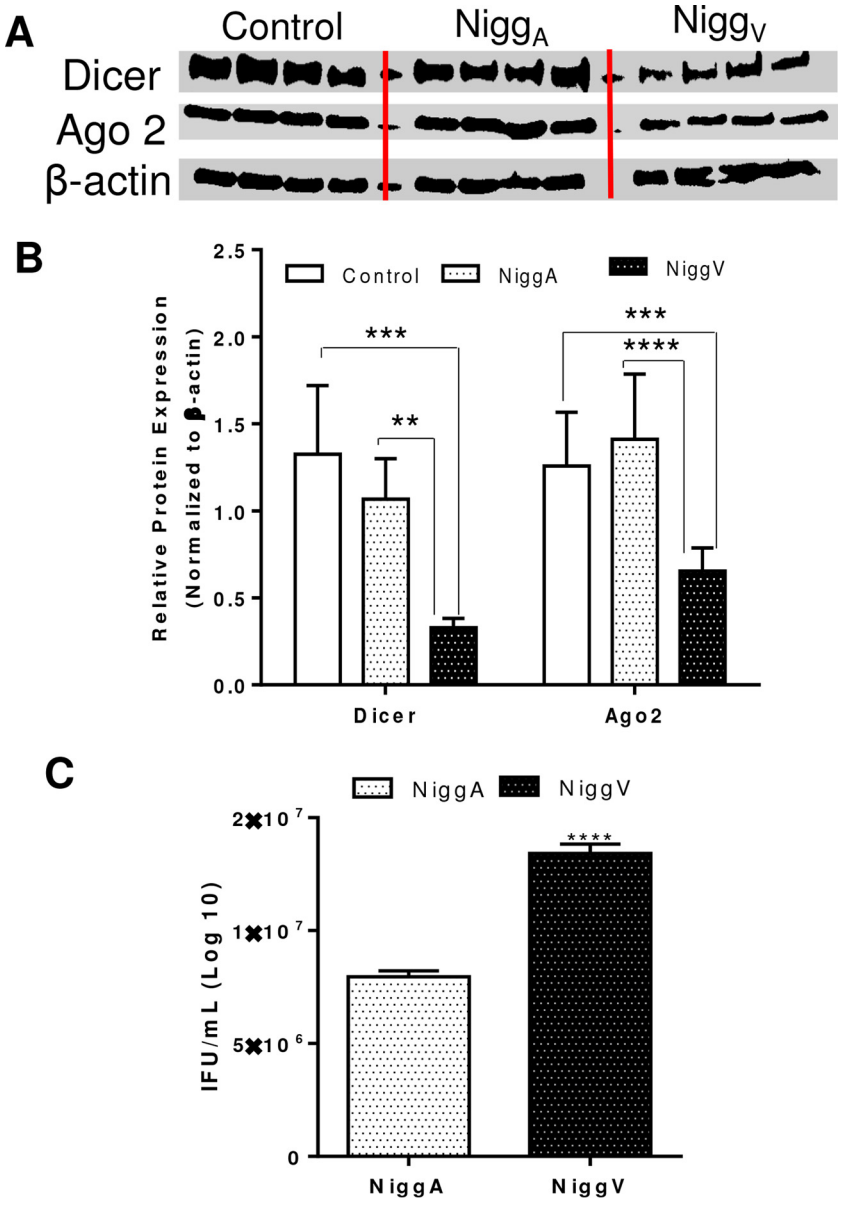

FIG 6 Dicer and Ago2 protein expression in epithelial cells infected with Nigg$_A$ or Nigg$_V$. (A) HeLa cells were infected with Nigg$_A$ or Nigg$_V$, and Dicer and Ago2 protein expression was determined by Western blotting analyses. A representative blot of two different experiments of Dicer and Ago2 with housekeeping protein β-actin is shown. Each lane represents replicates within the group ($n = 4$). (B) Densitometry analyses of data from two individual experiments are presented. Dicer and Ago2 expression was normalized to β-actin, and relative expression with respect to control is presented as mean $\pm$ SEM. The two experiments are statistically significant by themselves. Because of differences in band intensities from two experimental blots that were carried out at different times, the relative expression data are not on the same scale for Ago2 and Dicer. Thus, statistical significance was determined by 2-factor ANOVA, with experiment as a random factor and Tukey *post hoc* contrasts. The groups with significant differences are indicated (**, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). (C) IFU from Nigg$_A$- and Nigg$_V$-infected cell supernatants. Statistical significance was determined by two-tailed *t* test (****, $P < 0.0001$).

intavaginal model with Nigg$_A$ andNigg$_V$ strains. Thus, future studies will be designed to measure the miRNA response on day 4 with CmVar001 and CmVar004 variants, where a comparison with the Nigg$_A$ andNigg$_V$ infections will be possible. Overall, from our and other reports, detection of miRNA expression at early stages of infection could be associated with the inflammatory response and chronic pathology.

A previous report showed that miRNAs were enriched in pathways involved in

fibrosis and epithelial cell differentiation in human samples of chlamydial conjunctival inflammatory scarring (28). The study indicated that significant changes in miRNA expression are associated with the late-stage disease process. The miR-200 family has been shown to downregulate ZEB1/Zeb2 proteins, which are involved in fibrotic gene expression (47). Our data showed downregulation of miR-200b, miR-200b-5p, and 200b-3p miR-200a-3p, indicating higher expression of Zeb1/Zeb2 and increased fibrosis, in agreement with the previous report of Igietseme et al. (44). It is not surprising to see that these miRNAs were downregulated in both $Nigg_A$- and $Nigg_V$-infected mice, because some of the $Nigg_A$-infected animals also develop pathology. However, it would be interesting to determine if these miRNA responses persist in $Nigg_A$-infected mice at later time points. Moreover, upregulation of profibrotic miR-142-3p and miRNA 147 were also observed with $Nigg_V$ infection, consistent with the inflammatory *C. trachomatis* scarring model (28). $Nigg_V$ infection results in a higher bacterial load; thus, it is possible that increased inflammatory responses and miRNA expression are due to a higher replicative capacity of the chlamydiae. Interestingly, Gupta et al. (45) showed no overall differences in miRNA expression with three different doses (5×10^4, 5×10^5, and 5×10^6), suggesting that $Nigg_V$ interaction with host cells is different from that of $Nigg_A$ in terms of regulating host miRNA expression and is not necessarily related to numbers of microorganisms. Regardless of the replicative capacity of bacteria, our results suggest that miRNAs expression regulates the inflammatory response, thus dictating the pathological outcome.

miRNAs have been shown to regulate cytokine responses during infection with different pathogens (22). For example, peptidoglycan-mediated TLR2 signaling induces miR-132/-212 to downregulate IRAK4 and to prevent overproduction of proinflammatory cytokines (48). Interestingly, miR-132/-212 expression is significantly higher in $Nigg_V$-infected mice than in $Nigg_A$-infected mice. The data suggest that these miRNAs may prevent the overproduction of proinflammatory cytokines. miRNA-148a, miR-148b, and miR-152 have been shown to regulate the expression of I$\kappa\beta$-β, NF-$\kappa\beta$, IL-6, TNF-α, IL-12, IFN-β, MMP1, MMP14, and MMP16 (49), while we observed significantly higher levels of IL-6 and TNF-α in $Nigg_V$-infected mice. Taken together, the data suggest that upregulation of these miRNAs is important in controlling the expression of proinflammatory molecules. In addition, IL-1β represses miR-411 (50) expression, and we observed significantly lower miR-411 and higher IL-1β in $Nigg_V$-infected mice. Along similar lines, miR-100-5p represses IL-6 (51), and we observed lower levels of miR-100-5p and significantly more IL-6 expression in $Nigg_V$-infected mice than in $Nigg_A$-infected mice.

Finally, consistent with our results above, we found that $Nigg_V$ infection alters Dicer and Ago2 expression, while $Nigg_A$ infection has no significant impact on expression of these proteins (Fig. 6A and B). Previous studies showed that depletion of miRNA processing enzymes or increased expression of miRNA degradation exonucleases results in widespread decreases in miRNA expression (52). Simian immunodeficiency virus (SIV) infection led to a significant decrease in the expression of mucosal miRNA compared with that in uninfected controls, which correlated with an increase in 5′-3′-exoribonuclease 2 protein and alterations in Dicer1 and Argonaute 2 expression. Lower levels of Dicer were observed, which is also consistent with a previous report showing that *C. trachomatis* L2 infection cleaves Dicer in oviduct tissues of infertile mice (53). It is also possible that Dicer and Ago2 protein stability is impacted because of miRNAs abundance, as reported previously (16). Thus, further studies are warranted to determine if mRNA or protein degradation of Dicer and Ago2 contribute to the lower levels of these proteins. Furthermore, the expression of Ago2 and Dicer with the CmVar001 and CmVar004 variants seems to be similar (unpublished data) to NiggA and $Nigg_V$ data, indicating that virulent variants or strains impact the biogenesis machinery similarly. Our data further suggest that both canonical and noncanonical pathways involved in miRNA processing are likely impacted during $Nigg_V$ infection. From the current observations and our published study (3), we hypothesized that the more virulent *Chlamydia* strain alters the miRNA processing and/or degradation machinery

and thus regulates miRNA expression, which, in turn, could regulate chemokine/cytokine responses. Altered expression of Dicer and Ago2 during $Nigg_V$ infection likely contributes to the increased pathology observed with this virulent strain in addition to impacting miRNA expression. This is important because it suggests that more virulent chlamydiae have evolved a mechanism by which the inflammatory response is enhanced. As we have previously reported, polymorphonuclear leukocytes (PMNs) are intimately associated with the dislodgement of chlamydia-infected cells from the epithelium (42, 54, 55). The advantage to the microorganisms is that dislodgement of infected cells allows dispersal of the microorganisms so that new target cells may be infected. However, the consequences for the host are that ascension of the bacteria to the upper genital tract may result in infertility.

Based on the data, we conclude that infection with the more virulent $Nigg_V$ results in expression of miRNAs that regulate signal transduction pathways and fibrosis. The data demonstrate that fine-tuning of the inflammatory response is dependent on miRNAs expression. Future understanding of the role of each miRNA in regulating the inflammatory pathways would suggest targets for miRNA therapeutic intervention.

MATERIALS AND METHODS

Chlamydial strains. The *C. muridarum* Nigg strain has been continuously passaged in our laboratory since 1977, when it was obtained from the American Type Culture Collection as a yolk sac preparation. It was originally isolated from mice by Clara Nigg in 1942 as a natural respiratory infection of stock mice (56). As described above, it is a population of multiple genetic variants and has never been plaque purified. Interestingly, during a routine passage in 2014, it spontaneously developed into a more virulent stock as evidenced by its increased lethality during infection of immunodeficient $RAG^{-/-}$ mice (R. G. Rank and L. Yeruva, unpublished data). To differentiate these stocks, we have designated this new strain $Nigg_V$ and the parent strain $Nigg_A$. Both $Nigg_A$ and $Nigg_V$ stocks were cultured on McCoy cell monolayers. Stocks were stored in sucrose phosphate-buffered glutamic acid (SPG; 0.19 mM KH_2PO_4, 0.36 mM Na_2HPO_4,0.245 mM L-glutamic acid, 10.9 mM sucrose) at −80°C, and determination of IFU was carried out with HeLa cells.

Chlamydial genital tract infections. Seven-week-old C57BL/6 mice were given a 2-mg subcutaneous injection of progesterone (Depo-Provera; Upjohn, Kalamazoo, MI) 7 days prior to infection in order to place the mice in a state of anestrus (57). Mice were then infected intravaginally with 3×10^5 IFU (39), and swabs were collected once every 3 days until day 35. Chlamydiae were quantified from the genital tract using a Dacroswab and then cultured in HeLa cells according to standard procedure. Chlamydial inclusions were stained with *C. muridarum*-specific immune serum and with a fluorescent secondary anti-mouse IgG antibody (Alexa Fluor 488). On day 35, animals were euthanized, and gross pathology was noted by counting the oviducts for signs of hydrosalpinx. For *in vivo* chemokine, cytokine, and miRNA expression analyses, mice were infected intravaginally with 3×10^6 IFU, swabs were collected on day 3 for IFU enumeration, and genital tract tissues were collected on day 4. Animals that received SPG served as controls for data analyses. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences.

Chlamydial intranasal infections. Seven-week-old C57BL/6 mice were purchased from Jackson Laboratory and acclimated to the Arkansas Children's Research Institute (ACRI) animal facility for 7 days. Mice were anesthetized and placed in dorsal recumbence and then infected with a 10-μl volume containing 1×10^4 IFU of chlamydiae by placing the liquid over the nares in an alternating fashion. Mice were weighed prior to infection, and weight gain or loss was recorded daily.

Cell lines. HeLa or McCoy cells were cultured in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS), 1 mM glutamine, 100 μM nonessential amino acids (Invitrogen), and 50 μg/ml of gentamicin. For cytokine analyses, HeLa cells were plated in a 24-well dish (2×10^5 cells/well) and kept under culture conditions for 48 h prior to infection with $Nigg_A$ or $Nigg_V$. HeLa cells were infected at a multiplicity of infection (MOI) of 1, and cells were collected for RNA isolation at 24 h.

Total RNA isolation and expression analysis of miRNA and mRNA transcripts. Total RNA was isolated from cervices and cell samples using an RNeasy or miRNeasy kit (Qiagen). Briefly, cervical tissue was homogenized in Qiazol, and miRNA isolation was carried out according to the manufacturer's instructions. The concentration of RNA was determined by NanoDrop, and absorbances at 260 nm and 280 nm as well as 260 nm and 230 nm were measured to check for RNA quality. For chemokine/cytokine analyses, RNA was reverse transcribed with Superscript III enzyme (Invitrogen) according to the manufacturer's instructions using random hexamers and oligo(dT) for priming. Quantitative PCR was performed on samples using an IQ-SYBR mix (Bio-Rad) and CFX96 (Bio-Rad). Chlamydial rRNA (*rs*16) was measured to determine chlamydial growth. The concentrations of all the transcripts in each sample were normalized to the levels of actin and expressed as relative mRNA expression over that in the control (uninfected) group.

All primers were designed using Beacon Design software (Bio-Rad). To determine miRNA expression levels, 7 animals per group were used to run miRNA 4.0 chips from Affymetrix that contained mature murine miRNAs. We subjected the miRNA data to cluster analyses, an unbiased approach that allows preliminary examination of the data to know if the miRNA expression patterns (3,222 miRNAs) are similar

within the group. Data are presented as Venn diagram, heat map, and fold regulation (up or down) of miRNAs. To predict the target gene list of miRNAs expressed, Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, CA) was used, and a list of predicted target genes of the differentially expressed miRNAs which were either experimentally verified or predicted with high confidence was generated. The target gene list was subjected to canonical pathway analysis to identify significant functions or pathways being affected from a large set of known canonical pathways (apoptosis, cell cycle regulation, cellular growth and proliferation, cellular immune response, stress and injury, cytokine signaling, humoral immune response, organismal growth and development, pathogen-influenced signaling, and transcription regulation). The enrichment score was generated as a negative log *P* value based on the right-tailed Fisher's exact test (adjusted for false discovery rate at 5%) and is a measure of the likelihood of the association between the number of genes in our experiment and a given pathway. Furthermore, based on the microarray data, 10 miRNAs expression were confirmed by real-time PCR using Qiagen primer assays. Data were normalized to SNORD 68 and presented as expression relative to that of the control.

Western blotting. To determine if the difference in miRNA expression in the host during $Nigg_A$ or $Nigg_V$ infection is a result of modifications of the miRNA biogenesis machinery, we measured expression of key proteins, Ago2 and Dicer1, by Western blotting. HeLa cells were plated in a 6-well dish (4×10^6), cells were infected at 48 h with different chlamydial populations ($Nigg_A$ or $Nigg_V$) at an MOI of 1, and cells were collected at 24 h of infection. Uninfected cells served as controls. Supernatants were processed for IFU enumeration and cells were collected with 8 M urea containing 325 U/ml of Benzonase as described previously (58), sonicated for 60 s in a water sonicator, and then centrifuged at 12,000 $\times$ *g* for 15 min at 4°C. Supernatants were collected and the protein concentration was determined using a Bio-Rad protein estimation kit (Bio-Rad). Thirty micrograms of protein was run in a 6% acrylamide gel, membranes were probed overnight at 4°C with anti-human rabbit primary antibodies, and subsequently incubated with a 1:10,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody for 1 h at room temperature (Bio-Rad Laboratories Inc.). We used a 1:1,000 dilution of anti-Dicer (Sigma) and anti-Ago2 (Abcam) primary antibodies. Detection was performed using a chemiluminescence system (SuperSignal West chemiluminescent substrate; Thermo Scientific). ImageQuant software (ImageQuant TL 8.1) was used for densitometric analysis. Anti-β-actin antibody (Sigma-Aldrich) was used as a housekeeping protein for normalization of target protein.

Statistical analyses. For intranasal data and course of infection data, 2-factor (days, group) analysis of variance with repeated measures was carried out. Chi-square analyses with Yates correction was carried out for gross pathology data. Statistical significance of cytokine data was determined by two-tailed *t* test. For miRNA data analysis, microarray data were $\log_2$ transformed, centered, and scaled. miRNAs that differed significantly among the three groups were determined using the Kruskal-Wallis test. Pairwise comparisons between the groups were done by Mann-Whitney U test. Multiple-comparison adjustments were made by the Benjamini-Hochberg (BH) method ($P < 0.05$). Statistical significance for Western blotting and miRNA real-time PCR data was determined by one-way ANOVA with *post hoc* test. All the data with *P* values of <0.05 were considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00768-16.

TEXT S1, PDF file, 0.2 MB.

ACKNOWLEDGMENTS

This research was supported by the Arkansas Children's Research Institute, Arkansas Biosciences Institute, and CUMG (L.Y. and R.G.R.).

REFERENCES

1. Anestad G, Lunde O, Moen M, Dalaker K. 1987. Infertility and chlamydial infection. Fertil Steril 48:787–790. https://doi.org/10.1016/S0015-0282(16)59531-4.
2. Brunham RC, Maclean IW, Binns B, Peeling RW. 1985. Chlamydia trachomatis: its role in tubal infertility. J Infect Dis 152:1275–1282. https://doi.org/10.1093/infdis/152.6.1275.
3. Yeruva L, Myers GS, Spencer N, Creasy HH, Adams NE, Maurelli AT, McChesney GR, Cleves MA, Ravel J, Bowlin A, Rank RG. 2014. Early microRNA expression profile as a prognostic biomarker for the development of pelvic inflammatory disease in a mouse model of chlamydial genital infection. mBio 5:e01241-14. https://doi.org/10.1128/mBio.01241-14.
4. Lee RC, Feinbaum RL, Ambros V. 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75:843–854. https://doi.org/10.1016/0092-8674(93)90529-Y.
5. Denli AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ. 2004. Processing of primary microRNAs by the Microprocessor complex. Nature 432:231–235. https://doi.org/10.1038/nature03049.
6. Gregory RI, Yan KP, Amuthan G, Chendrimada T, Doratotaj B, Cooch N, Shiekhattar R. 2004. The Microprocessor complex mediates the genesis of microRNAs. Nature 432:235–240. https://doi.org/10.1038/nature03120.
7. Ohtsuka M, Ling H, Doki Y, Mori M, Calin GA. 2015. MicroRNA processing and human cancer. J Clin Med 4:1651–1667. https://doi.org/10.3390/jcm4081651.
8. Ha TY. 2011. The role of microRNAs in regulatory T cells and in the immune response. Immune Netw 11:11–41. https://doi.org/10.4110/in.2011.11.1.11.
9. Dai R, Ahmed SA. 2011. MicroRNA, a new paradigm for understanding immunoregulation, inflammation, and autoimmune diseases. Transl Res 157:163–179. https://doi.org/10.1016/j.trsl.2011.01.007.
10. O'Connell RM, Rao DS, Baltimore D. 2012. microRNA regulation of inflammatory responses. Annu Rev Immunol 30:295–312. https://doi.org/10.1146/annurev-immunol-020711-075013.
11. Estella C, Herrer I, Moreno-Moya JM, Quinonero A, Martinez S, Pellicer A,

Simon C. 2012. miRNA signature and Dicer requirement during human endometrial stromal decidualization in vitro. PLoS One 7:e41080. https://doi.org/10.1371/journal.pone.0041080.
12. Gonzalez G, Behringer RR. 2009. Dicer is required for female reproductive tract development and fertility in the mouse. Mol Reprod Dev 76:678–688. https://doi.org/10.1002/mrd.21010.
13. Hawkins SM, Andreu-Vieyra CV, Kim TH, Jeong JW, Hodgson MC, Chen R, Creighton CJ, Lydon JP, Gunaratne PH, DeMayo FJ, Matzuk MM. 2012. Dysregulation of uterine signaling pathways in progesterone receptor-Cre knockout of dicer. Mol Endocrinol 26:1552–1566. https://doi.org/10.1210/me.2012-1042.
14. Hong X, Luense LJ, McGinnis LK, Nothnick WB, Christenson LK. 2008. Dicer1 is essential for female fertility and normal development of the female reproductive system. Endocrinology 149:6207–6212. https://doi.org/10.1210/en.2008-0294.
15. Nagaraja AK, Andreu-Vieyra C, Franco HL, Ma L, Chen R, Han DY, Zhu H, Agno JE, Gunaratne PH, DeMayo FJ, Matzuk MM. 2008. Deletion of Dicer in somatic cells of the female reproductive tract causes sterility. Mol Endocrinol 22:2336–2352. https://doi.org/10.1210/me.2008-0142.
16. Martinez NJ, Gregory RI. 2013. Argonaute2 expression is post-transcriptionally coupled to microRNA abundance. RNA 19:605–612. https://doi.org/10.1261/rna.036434.112.
17. Winter J, Diederichs S. 2011. Argonaute proteins regulate microRNA stability: increased microRNA abundance by Argonaute proteins is due to microRNA stabilization. RNA Biol 8:1149–1157. https://doi.org/10.4161/rna.8.6.17665.
18. Xiao B, Liu Z, Li BS, Tang B, Li W, Guo G, Shi Y, Wang F, Wu Y, Tong WD, Guo H, Mao XH, Zou QM. 2009. Induction of microRNA-155 during *Helicobacter pylori* infection and its negative regulatory role in the inflammatory response. J Infect Dis 200:916–925. https://doi.org/10.1086/605443.
19. Izar B, Mannala GK, Mraheil MA, Chakraborty T, Hain T. 2012. microRNA response to *Listeria monocytogenes* infection in epithelial cells. Int J Mol Sci 13:1173–1185. https://doi.org/10.3390/ijms13011173.
20. Schulte LN, Eulalio A, Mollenkopf HJ, Reinhardt R, Vogel J. 2011. Analysis of the host microRNA response to Salmonella uncovers the control of major cytokines by the let-7 family. EMBO J 30:1977–1989. https://doi.org/10.1038/emboj.2011.94.
21. Qi Y, Cui L, Ge Y, Shi Z, Zhao K, Guo X, Yang D, Yu H, Cui L, Shan Y, Zhou M, Wang H, Lu Z. 2012. Altered serum microRNAs as biomarkers for the early diagnosis of pulmonary tuberculosis infection. BMC Infect Dis 12:384. https://doi.org/10.1186/1471-2334-12-384.
22. Singh RP, Massachi I, Manickavel S, Singh S, Rao NP, Hasan S, Mc Curdy DK, Sharma S, Wong D, Hahn BH, Rehimi H. 2013. The role of miRNA in inflammation and autoimmunity. Autoimmun Rev 12:1160–1165. https://doi.org/10.1016/j.autrev.2013.07.003.
23. Sarma NJ, Tiriveedhi V, Ramachandran S, Crippin J, Chapman W, Mohanakumar T. 2012. Modulation of immune responses following solid organ transplantation by microRNA. Exp Mol Pathol 93:378–385. https://doi.org/10.1016/j.yexmp.2012.09.020.
24. Shwetha S, Gouthamchandra K, Chandra M, Ravishankar B, Khaja MN, Das S. 2013. Circulating miRNA profile in HCV infected serum: novel insight into pathogenesis. Sci Rep 3:1555.
25. Wang K, Zhang S, Marzolf B, Troisch P, Brightman A, Hu Z, Hood LE, Galas DJ. 2009. Circulating microRNAs, potential biomarkers for drug-induced liver injury. Proc Natl Acad Sci U S A 106:4402–4407. https://doi.org/10.1073/pnas.0813371106.
26. Ha TY. 2011. MicroRNAs in human diseases: from lung, liver and kidney diseases to infectious disease, sickle cell disease and endometrium disease. Immune Netw 11:309–323. https://doi.org/10.4110/in.2011.11.6.309.
27. Ha TY. 2011. MicroRNAs in human diseases: from autoimmune diseases to skin, psychiatric and neurodegenerative diseases. Immune Netw 11:227–244. https://doi.org/10.4110/in.2011.11.5.227.
28. Derrick T, Roberts C, Rajasekhar M, Burr SE, Joof H, Makalo P, Bailey RL, Mabey DC, Burton MJ, Holland MJ. 2013. Conjunctival microRNA expression in inflammatory trachomatous scarring. PLoS Negl Trop Dis 7:e2117. https://doi.org/10.1371/journal.pntd.0002117.
29. Miyairi I, Mahdi OS, Ouellette SP, Belland RJ, Byrne GI. 2006. Different growth rates of *Chlamydia trachomatis* biovars reflect pathotype. J Infect Dis 194:350–357. https://doi.org/10.1086/505432.
30. Kari L, Whitmire WM, Carlson JH, Crane DD, Reveneau N, Nelson DE, Mabey DC, Bailey RL, Holland MJ, McClarty G, Caldwell HD. 2008. Pathogenic diversity among *Chlamydia trachomatis* ocular strains in nonhuman primates is affected by subtle genomic variations. J Infect Dis 197:449–456. https://doi.org/10.1086/525285.
31. Sturdevant GL, Kari L, Gardner DJ, Olivares-Zavaleta N, Randall LB, Whitmire WM, Carlson JH, Goheen MM, Selleck EM, Martens C, Caldwell HD. 2010. Frameshift mutations in a single novel virulence factor alter the in vivo pathogenicity of *Chlamydia trachomatis* for the female murine genital tract. Infect Immun 78:3660–3668. https://doi.org/10.1128/IAI.00386-10.
32. Jasper DK, Sigar IM, Schripsema JH, Sainvil CK, Smith CL, Yeruva L, Rank RG, Murthy AK, Widder JR, Ramsey KH. 2015. Genomic variant representation in a Chlamydia population is dynamic and adaptive with dependence on in vitro and in vivo passage. Pathog Dis 73:1–12. https://doi.org/10.1093/femspd/ftv003.
33. Chen C, Zhou Z, Conrad T, Yang Z, Dai J, Li Z, Wu Y, Zhong G. 2015. In vitro passage selects for Chlamydia muridarum with enhanced infectivity in cultured cells but attenuated pathogenicity in mouse upper genital tract. Infect Immun 83:1881–1892. https://doi.org/10.1128/IAI.03158-14.
34. Ramsey KH, Sigar IM, Schripsema JH, Denman CJ, Bowlin AK, Myers GSA, Rank RG. 2009. Strain and virulence diversity in the mouse pathogen *Chlamydia muridarum*. Infect Immun 77:3284–3293. https://doi.org/10.1128/IAI.00147-09.
35. Murthy AK, Li W, Chaganty BK, Kamalakaran S, Guentzel MN, Seshu J, Forsthuber TG, Zhong G, Arulanandam BP. 2011. Tumor necrosis factor alpha production from CD8+ T cells mediates oviduct pathological sequelae following primary genital Chlamydia muridarum infection. Infect Immun 79:2928–2935. https://doi.org/10.1128/IAI.05022-11.
36. Nagarajan UM, Prantner D, Sikes JD, Andrews CW, Jr, Goodwin AM, Nagarajan S, Darville T. 2008. Type I interferon signaling exacerbates *Chlamydia muridarum* genital infection in a murine model. Infect Immun 76:4642–4648. https://doi.org/10.1128/IAI.00629-08.
37. Nagarajan UM, Sikes JD, Yeruva L, Prantner D. 2012. Significant role of IL-1 Signaling, but limited role of inflammasome activation, in oviduct pathology during Chlamydia muridarum genital infection. J Immunol 188:2866–2875. https://doi.org/10.4049/jimmunol.1103461.
38. Manam S, Thomas JD, Li W, Maladore A, Schripsema JH, Ramsey KH, Murthy AK. 2015. TNF receptor 2 on CD8+ T cells and TNF receptor 1 on non-CD8+ T cells contribute significantly to upper genital tract pathology following chlamydial infection. J Infect Dis 211:2014–2022. https://doi.org/10.1093/infdis/jiu839.
39. Murthy AK, Chambers JP, Meier PA, Zhong G, Arulanandam BP. 2007. 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 75:666–676. https://doi.org/10.1128/IAI.01280-06.
40. Dong X, Liu Y, Chang X, Lei L, Zhong G. 2014. Signaling via tumor necrosis factor receptor 1 but not Toll-like receptor 2 contributes significantly to hydrosalpinx development following Chlamydia muridarum infection. Infect Immun 82:1833–1839. https://doi.org/10.1128/IAI.01668-13.
41. Darville T, O'Neill JM, Andrews CW, Jr, Nagarajan UM, Stahl L, Ojcius DM. 2003. Toll-like receptor-2, but not toll-like receptor-4, is essential for development of oviduct pathology in chlamydial genital tract infection. J Immunol 171:6187–6197. https://doi.org/10.4049/jimmunol.171.11.6187.
42. Lacy HM, Bowlin AK, Hennings L, Scurlock AM, Nagarajan UM, Rank RG. 2011. Essential role for neutrophils in pathogenesis and adaptive immunity in *Chlamydia caviae* ocular infections. Infect Immun 79:1889–1897. https://doi.org/10.1128/IAI.01257-10.
43. Derrick T, Last AR, Burr SE, Roberts CH, Nabicassa M, Cassama E, Bailey RL, Mabey DC, Burton MJ, Holland MJ. 2016. Inverse relationship between microRNA-155 and -184 expression with increasing conjunctival inflammation during ocular Chlamydia trachomatis infection. BMC Infect Dis 16:60.
44. Igietseme JU, Omosun Y, Stuchlik O, Reed MS, Partin J, He Q, Joseph K, Ellerson D, Bollweg B, George Z, Eko FO, Bandea C, Liu H, Yang G, Shieh WJ, Pohl J, Karem K, Black CM. 2015. Role of epithelial-mesenchyme transition in chlamydia pathogenesis. PLoS One 10:e0145198. https://doi.org/10.1371/journal.pone.0145198.
45. Gupta R, Arkatkar T, Yu JJ, Wali S, Haskins WE, Chambers JP, Murthy AK, Bakar SA, Guentzel MN, Arulanandam BP. 2015. Chlamydia muridarum infection associated host microRNAs in the murine genital tract and

contribution to generation of host immune response. Am J Reprod Immunol 73:126–140. https://doi.org/10.1111/aji.12281.
46. Gupta R, Arkatkar T, Keck J, Koundinya GK, Castillo K, Hobel S, Chambers JP, Yu JJ, Guentzel MN, Aigner A, Christenson LK, Arulanandam BP. 20 August 2016. Antigen specific immune response in Chlamydia muridarum genital infection is dependent on murine microRNAs-155 and -182. Oncotarget https://doi.org/10.18632/oncotarget.11461.
47. Chen Y, Ge W, Xu L, Qu C, Zhu M, Zhang W, Xiao Y. 2012. miR-200b is involved in intestinal fibrosis of Crohn's disease. Int J Mol Med 29: 601–606.
48. Nahid MA, Yao B, Dominguez-Gutierrez PR, Kesavalu L, Satoh M, Chan EK. 2013. Regulation of TLR2-mediated tolerance and cross-tolerance through IRAK4 modulation by miR-132 and miR-212. J Immunol 190: 1250–1263. https://doi.org/10.4049/jimmunol.1103060.
49. Liu X, Zhan Z, Xu L, Ma F, Li D, Guo Z, Li N, Cao X. 2010. MicroRNA-148/152 impair innate response and antigen presentation of TLR-triggered dendritic cells by targeting CaMKIIalpha. J Immunol 185:7244–7251. https://doi.org/10.4049/jimmunol.1001573.
50. Wang G, Zhang Y, Zhao X, Meng C, Ma L, Kong Y. 2015. MicroRNA-411 inhibited matrix metalloproteinase 13 expression in human chondrocytes. Am J Transl Res 7:2000–2006.
51. Aungier SR, Ohmori H, Clinton M, Mabbott NA. 2015. MicroRNA-100-5p indirectly modulates the expression of Il6, Ptgs1/2 and Tlr4 mRNA in the mouse follicular dendritic cell-like cell line, FL-Y. Immunology 144: 34–44. https://doi.org/10.1111/imm.12342.
52. Gaulke CA, Porter M, Han YH, Sankaran-Walters S, Grishina I, George MD, Dang AT, Ding SW, Jiang G, Korf I, Dandekar S. 2014. Intestinal epithelial barrier disruption through altered mucosal microRNA expression in human immunodeficiency virus and simian immunodeficiency virus infections. J Virol 88:6268–6280. https://doi.org/10.1128/JVI.00097-14.
53. Igietseme JU, Omosun Y, Partin J, Goldstein J, He Q, Joseph K, Ellerson D, Ansari U, Eko FO, Bandea C, Zhong G, Black CM. 2013. Prevention of Chlamydia-induced infertility by inhibition of local caspase activity. J Infect Dis 207:1095–1104. https://doi.org/10.1093/infdis/jit009.
54. Lee HY, Schripsema JH, Sigar IM, Lacy SR, Ramsey KH. 2010. A link between neutrophils and chronic disease manifestations of *Chlamydia muridarum* urogential infection of mice. FEMS Immunol Med Microbiol 59:108–116. https://doi.org/10.1111/j.1574-695X.2010.00668.x.
55. Rank RG, Whittimore J, Bowlin AK, Wyrick PB. 2011. *In vivo* ultrastructural analysis of the intimate relationship between polymorphonuclear leukocytes and the chlamydial developmental cycle. Infect Immun 79: 3291–3301. https://doi.org/10.1128/IAI.00200-11.
56. Nigg C. 1942. An unidentified virus which produces pneumonia and systemic infection in mice. Science 95:49–50.
57. Barron AL, White HJ, Rank RG, Soloff BL, Moses EB. 1981. A new animal model for the study of *Chlamydia trachomatis* genital infections: infection of mice with the agent of mouse pneumonitis. J Infect Dis 143: 63–66. https://doi.org/10.1093/infdis/143.1.63.
58. Chen AL, Johnson KA, Lee JK, Sutterlin C, Tan M. 2012. CPAF: a chlamydial protease in search of an authentic substrate. PLoS Pathog 8:e1002842. https://doi.org/10.1371/journal.ppat.1002842.