Intranasal Vaccination with *Chlamydia pneumoniae* Induces Cross-Species Immunity against Genital *Chlamydia muridarum* Challenge in Mice

Srikanth Manam1*, Bharat K. R. Chaganty2*, Shankar Jaikishan Evani2, Mark T. Zafiratos1, Anand K. Ramasubramanian2, Bernard P. Arulanandam2, Ashlesh K. Murthy1*

1 Department of Pathology, Midwestern University, Downers Grove, Illinois, United States of America, 2 South Texas Center for Emerging Infectious Diseases, Department of Biology, The University of Texas at San Antonio, San Antonio, Texas, United States of America

Abstract

*Chlamydia trachomatis* is the most common bacterial sexually transmitted disease in the world and specifically in the United States, with the highest incidence in age-groups 14–19 years. In a subset of females, the *C. trachomatis* genital infection leads to serious pathological sequelae including pelvic inflammatory disease, ectopic pregnancy, and infertility. *Chlamydia pneumoniae*, another member of the same genus, is a common cause of community acquired respiratory infection with significant number of children aged 5–14 yr displaying sero-conversion. Since these bacteria share several antigenic determinants, we evaluated whether intranasal immunization with live *C. pneumoniae* (1 × 10⁶ inclusion forming units; IFU) in 5 week old female C57BL/6 mice would induce cross-species protection against subsequent intravaginal challenge with *Chlamydia muridarum* (5 × 10⁷ IFU), which causes a similar genital infection and pathology in mice as *C. trachomatis* in humans. Mice vaccinated intranasally with live *C. pneumoniae*, but not mock (PBS) immunized animals, displayed high levels of splenic cellular antigen-specific IFN-γ production and serum antibody response against *C. muridarum* and *C. trachomatis*. Mice vaccinated with *C. pneumoniae* displayed a significant reduction in the vaginal *C. muridarum* shedding as early as day 12 after secondary i.vag. challenge compared to PBS (mock) immunized mice. At day 19 after *C. muridarum* challenge, 100% of *C. pneumoniae* vaccinated mice had cleared the infection compared to none (0%) of the mock immunized mice, which cleared the infection by day 27. At day 80 after *C. muridarum* challenge, *C. pneumoniae* vaccinated mice displayed a significant reduction in the incidence (50%) and degree of hydrosalpinx compared to mock immunized animals (100%). These results suggest that respiratory *C. pneumoniae* infection induces accelerated chlamydial clearance and reduction of oviduct pathology following genital *C. muridarum* challenge, and may have important implications to the *C. trachomatis*-induced reproductive disease in humans.

Introduction

*Chlamydia trachomatis* is the leading cause of bacterial sexually transmitted infection (STI), with approximately 90 million new cases detected annually worldwide [1]. The greatest incidence of infection is in the 14–19 year age group [2]. The infection is easily treatable with available antimicrobials [1,3]. However, repeated infections with the same or a different serovar occur commonly [4]. According to the Centers for Disease Control (2004), approximately 20–40% of women with past history of *C. trachomatis* infection(s) in the lower genital tract have been reported to develop serious sequelae such as pelvic inflammatory disease (PID). Subsets of patients with PID develop complications such as ectopic pregnancy and tubal infertility.

It is unclear why the reproductive sequelae develop only in a subset of women who contract chlamydial genital infections. Substantial research has been conducted to understand the immunity and pathogenesis relating to chlamydial STI using intravaginal *C. muridarum* infection in a mouse model that reasonably mimics the genital *C. trachomatis* infection and pathogenesis in humans [1,3]. While natural immunity confers resistance to reinfection, at least a subset of immune responses has been shown to be instrumental in the causation of pathologies. For example, several factors including activation of toll-like receptor-2 [5], neutrophil and matrix metalloprotease responses [6,7], and CD8+ T cells and TNF-α production [8] have been shown to contribute to the development of chlamydial pathological sequelae in the mouse model. Thus, the development of varying degrees of immune responses among individuals in a population, and pathological responses specifically, may determine the development of disease sequelae in some but not all infected women. Furthermore, chlamydial STI may relate spontaneously after several months due to natural immunity, or be detected and treated early with antimicrobials [1]. In the former scenario, a
robust immune response may act as a double-edged sword, assisting in the spontaneous clearance of infection while also promoting development of pathologies [9]. This line of thought has led to an emphasis on the induction of a protective, while eliminating pathogenic, immune response for anti-chlamydial vaccine development [10]. The latter scenario supports the “arrested immunity” hypothesis [11], which states that early detection and treatment of the infection prevents the development of a robust immune response. The consequences may be two-fold; increased susceptibility to infection upon subsequent exposure, and reduced propensity to develop pathological sequelae. In fact, the introduction of robust screening programs for chlamydial STI has been associated with dramatically increased prevalence rates of genital chlamydial infections and a parallel reduction in the prevalence of Chlamydia-associated PID [11]. Irrespective, both these scenarios are supported by the principle that host immune response against chlamydial infection is capable of inducing protective immunity, but also contributes to pathogenesis.

While the aforementioned scenarios are fully plausible and likely contribute, we suggest that an additional distinct variable typically operational in human populations may be capable of affecting the course of C. trachomatis infection and pathological sequelae in human populations. Chlamydia pneumoniae is the causative agent of respiratory disease in humans and one of the major causes of community-acquired pneumonia [12]. Beginning with a low prevalence in children under 5 years age, the prevalence increases dramatically to over 40% between 5–14 years age [12]. Approximately 50% of persons at age 20, and 75% of elderly have detectable antibody against C. pneumoniae. Importantly, a significant proportion of children display antibodies against C. pneumoniae before the onset of sexual activity and exposure to C. trachomatis STI. Comparative analysis of the genomes has revealed a very high degree of synteny between C. pneumoniae and C. trachomatis genomes [13], suggesting the likelihood of several shared antigenic determinants. Therefore, the immune responses induced by one pathogen may mediate cross-species protective immunity or pathogenic responses against the other. Therefore, we hypothesized that intranasal immunization with live C. pneumoniae will affect the course of infection and pathological sequelae following genital C. trachomatis challenge.

We tested this hypothesis in the mouse model by comparing the effect of intranasal live C. pneumoniae AR39 immunization on the course of vaginal infection and oviduct pathological sequelae following intravaginal C. muridarum challenge in C57BL/6 mice.

**Materials and Methods**

**Ethics Statement**

All animal experiments were performed in compliance with the Animal Welfare Act, the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and “Guide for the Care and Use of Laboratory Animals” published by the National Research Council. Animal work was done in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee (IACUC) at Midwestern University (Animal welfare assurance number A3048-01 and IACUC protocol number 2038) and at The University of Texas at San Antonio (Animal welfare assurance number A3592-01 and IACUC protocol number MU012). The respective IACUC at Midwestern University and the University of Texas at San Antonio specifically approved this study.

**Bacteria**

Chlamydia muridarum Nigg and Chlamydia trachomatis serovar D were grown separately on confluent HeLa cell monolayers as described previously [14, 15]. Chlamydia pneumoniae was grown on confluent HEp-2 cell monolayers as described previously [16]. The infected cells were lysed by sonication and elementary bodies (EBs) purified on Renografin gradients. Aliquots of bacteria were stored at −70°C in sucrose–phosphate–glutamine (SPG) buffer.

**Mice**

Four-to-six week old female C57BL/6 mice were used for all experiments. The mice were purchased from National Cancer Institute (Bethesda, MD) and housed at Midwestern University (MWU) and The University of Texas at San Antonio (UTSA). Animal care and experimental procedures were performed at MWU and UTSA in compliance with the respective Institutional Animal Care and Use Committee guidelines.

**Intranasal Immunization**

Groups of mice were anesthetized on day 0 with inhalational 3% isoflurane and injected intranasally with 25 µl per mouse of 1 × PBS containing 1 × 10⁶ IU of C. pneumoniae AR39 (C. pne). One group of mice receiving PBS alone (mock) served as negative control for the experiments. In experiments to evaluate protective immunity against pathology, one group of mice receiving 0.5 × 10⁶ IFU of C. muridarum (C. mur) on day 0 only served as positive control. The usage of the respective inocula for C. pne and C. mur is consistent with well-established mouse models of intranasal infection with these pathogens [16, 17]. Additionally, one group of mice receiving i.n. live C. pne immunization followed by mock (PBS) genital challenge was used to evaluate any contribution of the immunization per se towards disease development in the genital tract.

**Splenic Cellular Antigen-specific Cytokine Responses**

Spleens were removed 14 days after initial intranasal immunization and single cell suspensions prepared. Collected splenocytes [10⁵/well] were incubated for 72 h with 10⁵ IFU of UV-inactivated C. mur or C. tra, or 1 µg of an unrelated antigen bovine serum albumin (BSA), or in media alone in 96-well culture plates. Supernatants were assayed for levels of IFN-γ using ELISA kits (eBioscience, San Diego, CA) according to manufacturer’s instructions. Absorbance at 630 nm was measured using a Multiskan FC ELISA microplate reader (Thermoscientific Corp, Rockford, IL).

**Detection of Antibody Levels by ELISA**

On day 50 following intranasal immunization, animals were bled, sera prepared and analyzed by ELISA as described previously [14]. Microtiter plates (96-well) were coated overnight with 10⁵ IFU per well of C. mur or C. tra, or 1 µg of an unrelated antigen BSA, or PBS alone in sodium bicarbonate buffer (pH 9.5). Serial dilutions of serum were added to wells followed by goat anti-mouse total Ig (Southern Biotech, Birmingham, AL). After washing, horse radish peroxidase substrate (Sigma, St. Louis, MO) was added for color development and absorbance (O.D.) at 630 nm monitored using a Multiskan FC ELISA microplate reader (Thermoscientific Corp, Rockford, IL). Reciprocal serum dilutions corresponding to 50% maximal binding were used to obtain titers.
Vaginal Infection, Determination of Chlamydial Shedding and UGT Pathology

We confirmed that no chlamydial organisms could be recovered from the lungs of either C. pne or C. mur infected mice at day 45 after the intranasal immunization (data not shown). Mice were challenged i.vag. with 3 × 10^4 IFU of C. muridarum 60 days following intranasal immunization. This was considered day 0 for the challenge infection. Ten and three days prior to intravaginal infection, mice were treated with 2.5 mg of Depo-Provera (Upjohn, Kalamazoo, MI). Vaginal swab material was collected at the indicated days after challenge and chlamydial enumeration conducted by plating swab material on HeLa cell monolayers followed by immunofluorescent staining [14]. On day 80 after challenge, mice were euthanized, the genital tracts removed, placed next to a standard metric ruler, photographed and the greatest cross-sectional diameter measured for each oviduct, and reported individually and as mean ± SEM in a group, as also described previously [8]. Based on previous studies, we have reported individually and as mean

Statistics

Sigma Stat (Systat Software Inc., San Jose, CA) was used to perform all tests of significance. ANOVA was used to compare between multiple groups for cytokine and antibody response, chlamydial shedding and dilatation of hydrosalpinx. For chlamydial shedding, ANOVA with all pairwise multiple comparison procedures (Holm-Sidak method) was used to compare multiple groups at each time-point. The number of mice shedding Chlamydia at each time point in a group was used to compare the incidence of hydrosalpinx. Fisher’s exact test also was used to compare the incidence of hydrosalpinx. P<0.05 was considered statistically significant. All experiments were repeated at least twice, and each experiment was analyzed independently. Where oviduct diameter data is shown as a composite of two experiments, the indicated significant difference holds true when the experiments are analyzed individually.

Results

Antigen-specific Immune Responses after Intranasal Live C. pneumoniae Immunization

Immunity against genital chlamydial infection has been shown to involve antigen-specific IFN-γ producing cellular responses and antibody production [1,3,18]. Therefore, we evaluated these immune responses after i.n. immunization with C. pne or C. mur. On day 14 after initial intranasal C. pne, C. mur, or PBS (mock) immunization, mice were euthanized; spleens were removed and single cell suspensions made. Splenocytes (10^6 cells/well) were stimulated for 72 h with C. mur or C. tra or with an unrelated antigen BSA, or incubated in media alone. As shown in Fig. 1, C. pne immunized mice displayed high levels of IFN-γ response against C. mur and C. tra. Mice immunized i.n. with C. mur displayed significantly greater IFN-γ production when stimulated in vitro with either C. mur or C. tra when compared to C. pne immunized mice. Minimal IFN-γ response was produced against chlamydial antigens by cells from mice immunized with PBS, and from cells obtained from C. pne, C. mur or PBS immunized groups incubated with the unrelated antigen BSA, or media alone.

Figure 1. Splenic cellular antigen-specific IFN-γ response following immunization. C57BL/6 mice (3 mice/group) were treated i.n. with C. pneumoniae (1 × 10^6 IFU/mouse), or C. muridarum (0.5 × 10^6 IFU/mouse) in 25 μl of PBS, or PBS alone (mock). On day 14, animals were euthanized and splenocytes were tested by ELISA for antigen-specific IFN-γ production against C. mur, C. tra, or the unrelated antigen BSA. Cells cultured in media alone were used to evaluate baseline cytokine production from isolated splenocytes. Results are expressed as mean ± SEM of IFN-γ production per culture condition. *Significant (P<0.05, ANOVA) differences in IFN-γ production from splenocytes of C. pne versus C. mur immunized mice upon stimulation with C. mur or C. tra. Results are representative of two independent experiments. doi:10.1371/journal.pone.0064917.g001

Splenocytes from C. pne immunized mice expectedly displayed significantly greater IFN-γ production upon stimulation with C. pne as compared to any other treatment (data not shown).

The serum total antibody responses also were measured against C. mur and C. tra at day 50 following the intranasal immunization. As shown in Fig. 2, high and comparable levels of cross-species anti-C. mur and anti-C. tra total Ab response was induced by both C. pne and C. mur immunization. There was minimal binding in plates coated with an unrelated antigen BSA, indicating the specificity of these responses against chlamydial antigens. Serum anti-C. pne total antibody levels expectedly were significantly greater than cross-reactive antibodies against C. mur or C. tra (data not shown). Collectively, these results demonstrate that i.n. immunization with live C. pne induce antigen specific IFN-γ and antibody response against C. muridarum and C. trachomatis.

Vaginal Bacterial Clearance following Chlamydial Challenge in Immunized Mice

The immunized animals were rested for one month following the i.n. immunization and challenged i.vag. with 5 × 10^4 IFU of C. muridarum. The vaginal chlamydial shedding was monitored on the indicated days for a period of 30 days following bacterial challenge. As shown in Fig. 3 and Table 1, PBS (mock) immunized mice displayed high levels of chlamydial shedding on day 4 after inoculation, and displayed progressive reduction in bacterial shedding, with complete resolution by day 27 after inoculation. C. pne immunized mice displayed comparable bacterial shedding on days 4 and 8, followed by significantly reduced shedding on day 12 and subsequent time periods when compared to mock immunized animals. All (100%) C. pne immunized mice displayed complete resolution of infection by day 19 after inoculation, and chlamydial shedding in this group was significantly reduced over the indicated days following bacterial challenge.
the course of infection when compared to mock immunized animals. As expected, C. mur immunized mice exhibited significantly reduced chlamydial shedding as early as day 4, and all mice had completely resolved the infection by day 8 after inoculation. C. mur immunized mice displayed significant reduction in shedding when compared to mock (PBS) immunized animals. As also expected, C. pneumoniae immunized and mock (PBS) challenged mice did not display shedding of either C. pneumoniae or C. mur from the vagina.

These results suggest that i.n. immunization with live C. pneumoniae induces reduced chlamydial shedding and early bacterial clearance following i. vag. C. muridarum challenge.

Upper Genital Tract Pathology following Challenge in Immunized Mice

The immunized/challenged mice were rested until day 80 after challenge and upper genital tract pathology was evaluated. Our previous extensive studies [8,14,18–21] have demonstrated the suitability of this time-period for analyses of upper genital tract sequelae induced by genital C. muridarum infection in mice. The macroscopic pathology was evaluated based on the presence of hydrosalpinx, and is reported as the percentage of mice displaying hydrosalpinx bilateral, unilateral, and total hydrosalpinx at day 80 after chlamydial challenge (Table 2). Hydrosalpinx was observed in 100% (33% bilateral, 67% unilateral) of mock (PBS) immunized mice. In comparison, C. pneumoniae immunized mice displayed significant reduction in the incidence of hydrosalpinx by 50% (17% bilateral; 33% unilateral). As shown also in several previous studies [3], pathology was minimal in C. mur immunized group and only 16% (8% bilateral; 8% unilateral) displayed hydrosalpinx. The incidence of hydrosalpinx in C. mur immunized group was significantly reduced from that in mock (PBS) immunized mice, but not from that in C. pneumoniae immunized group. Chlamydia pneumoniae immunized mice that were not challenged intravaginally with C. mur displayed normal oviducts, suggesting that the intranasal C. pneumoniae immunization per se did not affect the oviduct.

We also quantified the oviduct dilatation by taking measurements of the greatest cross-sectional diameter of each oviduct, since the degree of oviduct dilatation may be an indicator of the severity of pathology. As shown in Fig. 4, 75% of oviducts in PBS
immunized mice were dilated, and displayed a high level of dilatation. In comparison, significantly fewer (25%) oviducts were dilated in Chlamydia pneumoniae immunized mice, with a significant reduction in the degree of oviduct dilation when compared to mock (PBS) immunized animals. Additionally, C. muridarum immunized mice displayed further reduction, albeit not statistically different, in the incidence (12%) and degree of oviduct dilatation when compared to Chlamydia pneumoniae immunized animals. All Chlamydia pneumoniae immunized mice that were not challenged intravaginally with C. mur was displayed normal oviduct diameters. We analyzed these tissues further microscopically, but the cellular infiltration at 80 days after genital challenge was minimal (data not shown). Collectively, these results suggest that intranasal live C. pneumoniae immunization induces robust protective immunity, close to that induced by i.n. live C. mur immunization, against oviduct pathological sequelae following genital C. mur challenge.

Discussion

We provide evidence that intranasal infection with Chlamydia pneumoniae induces cross-species antigen-specific IFN-γ and antibody response against Chlamydia muridarum and Chlamydia trachomatis. Furthermore, the i.n. C. pneumoniae infection induces significantly accelerated chlamydial clearance, and significant reduction in the incidence and degree of oviduct pathological sequelae following i.vag. C. muridarum challenge.

Cross-reactive immune responses in Chlamydia pneumoniae immunized mice induced significant reduction in chlamydial shedding and earlier resolution of the i.vag. C. mur infection compared to mock (PBS) immunized animals. To this end, we found a high level of C. mur-specific IFN-γ production from splenocytes of mice immunized previously with Chlamydia pneumoniae. Although there was accelerated resolution of the intravaginal challenge infection, all (100%) of Chlamydia pneumoniae immunized mice got infected upon i.vag. C. mur challenge and shed high levels of C. mur at days 4 and 8 after challenge, comparable to mock (PBS) immunized mice. This was in contrast to mice immunized i.n. with C. mur which displayed significant early resistance to the challenge, with 67% displaying no chlamydial shedding as early as day 4, and all (100%) of the mice resolving the infection by day 8 after i.vag. challenge. The differences in the level of protective immunity observed between i.n. C. pneumoniae versus C. mur immunized groups correlated with the differences in levels of splenic antigen-specific IFN-γ production. The production of Chlamydia-specific IFN-γ has been shown to be crucial in mediating enhanced clearance and protective immunity against C. mur challenge [1,3,18].

The total antibody levels against either C. mur were comparable between mice immunized i.n. with C. pneumoniae or C. mur, and did not correlate with the differences in clearance or pathology following i.vag. C. mur challenge. However, previous reports from the mouse model of C. mur genital infection have suggested an important role for antibody in inducing early resistance to reinfection [22]. Among several chlamydial antigens evaluated till date as putative vaccine candidates, only antibody against the chlamydial major outer membrane protein (MOMP) has been shown to be efficacious in neutralizing chlamydial infectivity [23–25]. Additionally, it has been shown that MOMP-induced neutralizing efficacy is dependent on the three dimensional conformation of this protein [26]. MOMP is the basis of serovar differentiation within Chlamydia trachomatis, and expectedly MOMP displays significant variation in amino acid sequence between different species of

Table 1. Bacterial clearance after intravaginal C. muridarum challenge in immunized animals.

| i.n infection/i.vag. challenge | % of mice shedding Chlamydia from the vagina Days after i.vag. challenge |
|------------------------------|-------------------------------------------------|
|                              | 4      | 8      | 12     | 15     | 19     | 24     | 27     | 30     |
| PBS/C. mur                   | 100    | 100    | 100    | 100    | 100    | 33     | 0      | 0      |
| @ C. pneumoniae/C. mur       | 100    | 100    | 67     | 67     | 0      | 0      | 0      | 0      |
| C. mur/C. mur               | 33     | 0      | 0      | 0      | 0      | 0      | 0      | 0      |
| C. pneumoniae/PBS            | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      |

The percentage of mice within a group (n = 6) shedding Chlamydia at each time point was added over the entire monitoring period, and the sum was compared between groups.

*Significant (P < 0.05, Fisher’s exact test) differences between mouse groups immunized with PBS (mock) compared to C. pneumoniae and PBS (mock) compared to C. mur. Results are representative of two independent experiments.

doi:10.1371/journal.pone.0064917.t001

Table 2. The incidence of oviduct pathology after intravaginal C. muridarum challenge in immunized animals.

| i.n infection/i.vag. challenge | % of mice displaying hydrosalpinx Day 80 after i.vag. challenge |
|------------------------------|-------------------------------------------------|
|                              | B      | U      | T      |
| PBS/C. mur                   | 33     | 67     | 100    |
| C. pneumoniae/C. mur         | 17     | 33     | 50     |
| C. mur/C. mur                | 8      | 8      | 16     |
| C. pneumoniae/PBS            | 0      | 0      | 0      |

The number of mice within a group (n = 6) developing hydrosalpinx was compared between groups.

*Significant (P < 0.05, Fisher’s exact test) differences between mouse groups immunized with PBS (mock) compared to C. pneumoniae and PBS (mock) compared to C. mur. Results are a composite of two independent experiments and thus reflect 12 mice per group.

doi:10.1371/journal.pone.0064917.t002
Results are a composite of two independent experiments and thus reflect 24 oviducts per group. These results demonstrate that a respiratory C. pneumoniae challenge reduces the chlamydial shedding and oviduct pathology without affecting the incidence of infection upon i.vag. C. muridarum infection is capable of inducing a high level of anti-Chlamydia immunity. Specifically, (A) protection, albeit arguably lesser than that induced by a prior C. pneumoniae infection, against oviduct pathological sequelae in females following genital C. trachomatis infection. Furthermore, the demonstration of C. pneumoniae induced cross-species immune response against the highly prevalent C. trachomatis serovar D suggests the likelihood of a similar possibility in context of C. trachomatis STI in humans.

*Chlamydia* [27]. Therefore, even though the total antibody levels may be similar, varying levels of antibodies which neutralize infectivity may contribute to differences between i.n. C. pneumoniae versus C. muridarum immunized mice with respect to early resistance to i. vag. C. muridarum challenge.

We [14,18,21] and others [3] also have shown previously that the production of *Chlamydia*-specific IFN-γ also is crucial for the reduction of oviduct pathological sequelae following i.vag. C. muridarum challenge. In this study, we found that C. pneumoniae immunization induced a strong anti-C. muridarum IFN-γ response and a significant reduction in the incidence and severity of oviduct dilatation following i.vag. C. muridarum challenge. In comparison to C. pneumoniae immunization, intranasal immunization with live C. muridarum induced significantly greater anti-C. muridarum IFN-γ response and a further reduction, albeit not statistically significant, in oviduct pathology. These results demonstrate that a respiratory C. pneumoniae infection is capable of inducing a high level of protection, albeit arguably lesser than that induced by a prior C. muridarum infection, against oviduct pathological sequelae.

The results of this study provide a new perspective to our understanding of anti-chlamydial immunity. Specifically, (A) respiratory C. pneumoniae infection induces cross-species antigen-specific IFN-γ and antibody response against C. muridarum and C. trachomatis, and (B) respiratory C. pneumoniae infection induces significant reduction in the chlamydial shedding and oviduct pathology without affecting the incidence of infection upon i.vag. C. muridarum challenge. These should be considered in context of the observations that (C) intravaginal C. muridarum infection in mice is a reasonable model to study genital C. trachomatis infections in humans [1,3] and (D) a significant number of children who contract C. trachomatis STI have been previously exposed to respiratory C. pneumoniae infection [12]. Collectively, it appears that prior exposure to C. pneumoniae infection may be an important factor in humans which may, at least partially, affect the duration of bacterial shedding and the development of pathological sequelae in females following genital C. trachomatis infection. The confirmation of such a possibility in studies involving humans also will have important implications to the approaches used for anti-chlamydial vaccine development.

In summary, we have used the mouse model to demonstrate a role for antecedent respiratory C. pneumoniae infection in altering the course of and sequelae to subsequent intravaginal C. muridarum infection. Furthermore, the demonstration of C. pneumoniae induced cross-species immune response against the highly prevalent C. trachomatis serovar D suggests the likelihood of a similar possibility in context of C. trachomatis STI in humans.

**Author Contributions**

Conceived and designed the experiments: AKM. Performed the experiments: SM BKRC SJM MF. Analyzed the data: SM BKRC AKR BPA AKM. Contributed reagents/materials/analysis tools: BPA AKM. Wrote the paper: SM AKM.

Figure 4. The incidence and severity of oviduct pathology after intravaginal C. muridarum challenge in immunized animals. C57BL/6 mice (3 mice/group) were treated i.n. with C. pneumoniae (1 × 10^6 IFU/mouse), or C. muridarum (0.5 × 10^3 IFU/mouse) in 25 μl of PBS, or PBS alone (mock). The mice were challenged 60 days later intravaginally with 5 × 10^4 IFU of C. muridarum in 10 μl of PBS. One group of C. pneumoniae immunized mice was mock challenged intravaginally with 10 μl of PBS alone. Mice were euthanized, genital tract tissues collected, and macroscopic oviduct dilatation was measured on day 80 after challenge. Each individual marker represents one oviduct and the mean ± SEM of greatest cross-sectional oviduct diameter per group of mice is also shown. The number of normal oviducts (numerator) and the total number of oviducts evaluated (denominator), and the percentage of normal oviducts per respective group of mice have been indicated in parentheses. * Significant (P < 0.05, ANOVA) difference in the degree of oviduct dilatation in C. muridarum challenged mice immunized previously with PBS (mock) compared to C. pneumoniae, and between mice immunized previously with PBS (mock) compared to C. muridarum. # Significant (P < 0.05, Fisher’s exact test) difference in the incidence of oviduct dilatation in C. pneumoniae challenged mice immunized previously with PBS (mock) compared to C. pneumoniae, and in mice immunized previously with PBS (mock) compared to C. muridarum. Results are a composite of two independent experiments and thus reflect 24 oviducts per group.

doi:10.1371/journal.pone.0064917.g004
References

1. Brunham RC, Rey-Ladino J (2005) Immunology of Chlamydia infection: implications for a Chlamydia trachomatis vaccine. Nat Rev Immunol 5: 149–161.
2. Centers for Disease Control (2011) CDC Grand Rounds: Chlamydia Prevention: Challenges and Strategies for Reducing Disease Burden and Sequelae. Morbidity and Mortality Weekly Report 60(12): 370–372.
3. Morrison RP, Caldwell HD (2002) Immunity to murine chlamydial genital infection. Infect Immun 70: 2741–2751.
4. Kelly KA (2003) Cellular immunity and Chlamydia genital infection: induction, recruitment, and effector mechanisms. Int Rev Immunol 22: 3–41.
5. Daveille T, O’Neill JM, Andrews CW, Jr., Nagarajan UM, Stahl L, et al. (2003) Toll-like receptor-2, but not Toll-like receptor-4, is essential for development of oxidant pathology in chlamydial genital tract infection. J Immunol 171: 6187–6197.
6. Imtiaz MT, Schripsema JH, Sigar IM, Kasimos JN, Ramsey KH (2006) Inhibition of matrix metalloproteinases protects mice from ascending infection and chronic disease manifestations resulting from urogenital Chlamydia muridarum infection. Infect Immun 74: 5513–5521.
7. Imtiaz MT, Distelhorst JT, Schripsema JH, Sigar IM, Kasimos JN, et al. (2007) A role for matrix metalloproteinase-9 in pathogenesis of urogenital Chlamydia muridarum infection in mice. Microbes Infect 9: 1561–1566.
8. Murthy AK, Li W, Chaganty BK, Kamalakaran S, Guentzel MN, et al. (2011) Tumor necrosis factor alpha production from CD8+ T cells mediates oxidant pathological sequelae following primary genital Chlamydia muridarum infection. Infect Immun 79: 2928–2935.
9. Debatasta J, Timms P, Allan J, Allan J (2005) Immunopathogenesis of Chlamydia trachomatis infections in women. Fertil Steril 79: 1273–1287.
10. Rockey DD, Wang J, Lei L, Zhong G (2009) Chlamydia vaccine candidates and tools for chlamydial antigen discovery. Expert Rev Vaccines 8: 1365–1377.
11. Rekart ML, Brunham RC (2005) Immunology of Chlamydia pneumoniae infection. J Exp Med 197: 459–461.
12. Kuo CC, Jackson LA, Campbell LA, Grayston JT (1995) Chlamydia pneumoniae (TWAR). Clin Microbiol Rev 8: 431–461.
13. Read TD, Brunham RC, Shun G, Gill SR, Heidelberg JF, et al. (2000) Genome sequences of Chlamydia trachomatis Mu5On and Chlamydia pneumoniae AR39. Nucleic Acids Res 28: 1397–1406.
14. 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 oxidant pathology, and is highly dependent upon endogenous gamma interferon production. Infect Immun 75: 666–670.
15. Dong F, Zhong Y, Arulanandam B, Zhong G (2003) Production of a protoeuctically active protein, chlamydial protease/proteosome-like activity factor, by five different Chlamydia species. Infect Immun 71: 1868–1872.
16. Campbell LA, Nosaka T, Rosenfeld ME, Yarzeit K, Kuo CC (2005) Tumor necrosis factor alpha plays a role in the acceleration of atherosclerosis by Chlamydia pneumoniae in mice. Infect Immun 73: 3164–3165.
17. Yang X, Brunham RC (1998) Gene knockout B cell-deficient mice demonstrate that B cells play an important role in the initiation of T cell responses to Chlamydia trachomatis (mouse pneumonitis) lung infection. J Immunol 161: 1439–1446.
18. Murthy AK, Guentzel MN, Zhong G, Arulanandam BP (2009) Chlamydial protease-like activity factor–insights into immunity and vaccine development. J Reprod Immunol 83: 179–184.
19. Murthy AK, Li W, Guentzel MN, Zhong G, Arulanandam BP (2011) Vaccination with the defined chlamydial secreted protein CPAF induces robust protection against female infertility following repeated genital chlamydial challenge. Vaccine 29: 2519–2522.
20. Murthy AK, Chaganty BK, Li W, Guentzel MN, Chambers JP, et al. (2009) A limited role for antibody in protective immunity induced by rCPAF and CpG vaccination against primary genital Chlamydia muridarum challenge. FEMS Immunol Med Microbiol 55: 271–279.
21. Li W, Murthy AK, Guentzel MN, Sadas J, Forshuber TG, et al. (2006) Antigen-specific CD4+ T cells produce sufficient IFN-gamma to mediate robust protective immunity against genital Chlamydia muridarum infection. J Immunol 180: 3373–3382.
22. Morrison SG, Morrison RP (2005) A predominant role for antibody in acquired immunity to chlamydial genital tract reinfection. J Immunol 175: 7536–7542.
23. Caldwell HD, Perry LJ (1982) Neutralization of Chlamydia trachomatis infectivity with antibodies to the major outer membrane protein. Infect Immun 38: 756–764.
24. Pal S, Bravo J, Peterson EM, de la Maza LM (2008) Protection of wild-type and severe combined immunodeficiency mice against an intranasal challenge by passive immunization with monoclonal antibodies to the Chlamydia trachomatis major outer membrane protein. Infect Immun 76: 5501–5507.
25. Pal S, Peterson EM, Rappuoli R, Ratti G, de la Maza LM (2006) Immunization with the Chlamydia trachomatis major outer membrane protein, using adjuvants developed for human vaccines, can induce partial protection in a mouse model against a genital challenge. Vaccine 24: 766–775.
26. Pal S, Peterson EM, de la Maza LM (2005) Vaccination with the Chlamydia trachomatis major outer membrane protein can elicit an immune response as protective as that resulting from inoculation with live bacteria. Infect Immun 73: 6153–6160.
27. Stephens RS, Wagar EA, Schoolnik GK (1988) High-resolution mapping of serovar-specific and common antigenic determinants of the major outer membrane protein of Chlamydia trachomatis. J Exp Med 167: 817–831.