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Induction of protective immunity against *Chlamydia muridarum* intracervical infection in DBA/1j mice

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Abstract

We previously reported that intracervical inoculation with *Chlamydia muridarum* induced hydrosalpinx in DBA/1j mice, but intravaginal inoculation failed to do so. In the current study, we found unexpectedly that intrabursal inoculation of live chlamydial organisms via the oviduct failed to induce significant hydrosalpinx. We further tested whether primary infection via intravaginal or intrabursal inoculation could induce protective immunity against hydrosalpinx following intracervical challenge infection. Mice infected intravaginally with *C. muridarum* were fully protected from developing hydrosalpinx, while intrabursal inoculation offered partial protection. We then compared immune responses induced by the two genital tract inoculations. Both inoculations induced high IFN γ and IL-17 T cell responses although the ratio of IgG2a versus IgG1 in intravaginally infected mice was significantly higher than in mice infected intrabursally. When the antigen-specificities of antibody responses were compared, both groups of mice dominantly recognized 24 *C. muridarum* antigens, while each group preferentially recognized unique sets of antigens. Thus, we have demonstrated that intrabursal inoculation is neither effective for causing hydrosalpinx nor efficient in inducing protective immunity in DBA/1j mice. Intravaginal immunization, in combination with intracervical challenge infection in DBA/1j mice, can be a useful model for understanding mechanisms of chlamydial pathogenicity and protective immunity.

Keywords

Intrabursal; Intravaginal; Intracervical infection; *Chlamydia muridarum*; DBA/1J

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1. Introduction

Chlamydia trachomatis is a leading cause of sexually transmitted bacterial infections, which can lead to upper genital tract pathology such as hydrosalpinx [1]. Although chlamydial intracellular infection-induced inflammatory responses are thought to contribute significantly to chlamydial pathogenicity [2, 3], the precise mechanisms on how the lower genital tract infection ascends to the upper genital tract and how the ascending chlamydial organisms trigger pathological responses in the upper genital tract remain unknown. There is still no licensed anti-*C. trachomatis* vaccine. *Chlamydia muridarum* organisms have been extensively used to study the mechanisms of *C. trachomatis* pathogenesis and immunity [4] although *C. muridarum* causes no known human diseases. Intravaginal inoculation of mice with *C. muridarum* can lead to hydrosalpinx, which closely mimics the tubal pathology induced by *C. trachomatis* in humans [5]. Hydrosalpinx has been used as a surrogate marker for tubal occlusion and tubal factor infertility [5-7]. Intrabursal inoculation with *C. muridarum* can induce infertility in some strains of mice, which has been successfully used for evaluating chlamydial vaccine candidate antigens [8, 9].

We previously reported that DBA/1j mice were highly resistant to hydrosalpinx induction by intravaginal infection with *C. muridarum*. However, intracervical inoculation effectively overcame the resistance, which correlates the resistance to hydrosalpinx with insufficient ascending infection into the upper genital tract [10]. Since intrabursal inoculation involves direct inoculation of live chlamydial organisms into the upper genital tract, we evaluated whether intrabursal inoculation can induce hydrosalpinx in DBA/1j mice. To our surprise, intrabursal inoculation, like intravaginal inoculation, failed to induce significant hydrosalpinx. We then tested whether immunization via intravaginal or intrabursal inoculation could induce protective immunity against hydrosalpinx induced by intracervical challenge infection. Mice infected intravaginally with *C. muridarum* were fully protected from developing hydrosalpinx while infection via intrabursal inoculation only offered partial protection. In terms of immune responses induced by the two genital tract mucosal inoculations, both inoculations induced Th1- and Th17-dominant responses and robust *C. muridarum*-specific antibody responses. Nevertheless, each group preferentially recognized unique sets of antigens. These observations have provided important information for understanding the mechanisms of chlamydial pathogenesis and also revealed that intravaginal immunization in combination with intracervical challenge infection in DBA/1j mice can be a useful model for evaluating vaccine efficacy.

2. Materials and Methods

2.1. Chlamydial organisms and infection

The *C. muridarum* organisms (Nigg strain) used in the current study were propagated in HeLa cells (human cervical carcinoma epithelial cells, ATCC cat# CCL2.1), purified, aliquoted and stored as described previously [3, 11]. Female DBA/1j (000670) were purchased at the age of 5 to 6 weeks old from Jackson Laboratories (Bar Harbor, Maine). Each mouse was inoculated intravaginally with 2×10^5 IFUs of live *C. muridarum* organisms in 20 μ l of SPG (sucrose-phosphate-glutamate buffer), intrabursally with the same amount of organisms in 10 μ l or intracervically in 3 μ l of SPG. Five days prior to any inoculation, each mouse was injected subcutaneously with 2.5mg Depo-provera (Pharmacia Upjohn, Kalamazoo, MI). For intravaginal inoculation, the inoculum was delivered into mouse vagina using a 200 μ l micropipette tip as described previously [3]. For intracervical inoculation, a Non-Surgical Embryo Transfer Device (NSET, cat# 60010, ParaTechs Corp., Lexington, KY) was used and the manufacturer's instruction (<http://www.paratechs.com/nset/>) was followed as described previously [10]. The intrabursal infection was carried out based on a protocol described previously [8, 12]. Briefly, mice were anesthetized and laid

with the dorsal side up on a sterile gauze pad with the mouse head facing away. A small incision was made at the dorsomedial position and directly above the ovarian fat pad. After the ovarian fat pad was gently pulled out, the ovary was positioned to allow for insertion of a needle (30GA, Removable needles, Hamilton, #7803-07) into the oviduct tubule. When the needle was inserted into the proper position, it was visible under the bursa. The plunger of the syringe (Hamilton, #7654-01) was gently pushed to inject the 10 μ l of inoculum, after which the needle was quickly removed and the puncture site was gently sealed. The bursa should be slightly distended if the injection is successful. Finally, the reproductive tract and fat pad were gently put back into the peritoneal cavity and the body wall was closed and sutured (Reli sutures, SK683, Busan, South Korea). After recovery, the mice were returned to cages for normal care. For *in vitro* infection, HeLa cells grown on coverslips in 24-well plates containing DMEM (GIBCO BRL, Rockville, MD) with 10% fetal calf serum (FCS; GIBCO BRL) at 37°C in an incubator supplied with 5% CO₂ were inoculated with *C. muridarum* organisms as described previously [3, 13]. The infected cultures were examined by immunofluorescence as described below.

2.2. Monitoring live *C. muridarum* organism recovery from swab samples

To monitor live organism shedding, vaginal swabs were taken on different days after intravaginal or intrabursal inoculation or intracervical challenge infection. Each swab was suspended in 500 μ l of ice-cold SPG followed by vortexing with glass beads, and the released organisms were titrated on HeLa cell monolayers in duplicates as described previously [3]. The total number of inclusion forming units (IFUs) per swab was calculated based on the number of IFUs per field, number of fields per coverslip, dilution factors and inoculation and total sample volumes. An average was taken from the serially diluted and duplicate assays for any given swab. The calculated total number of IFUs/swab was converted into log₁₀, and the log₁₀ IFUs were used to calculate means and standard deviation for each group at each time point.

2.3. Evaluating mouse genital tract tissue pathologies and histological scoring

Mice were sacrificed on day 60 after intravaginal or intrabursal inoculations or intracervical challenge infection, and the mouse urogenital tract tissues were isolated. Before the tissues were removed, an *in situ* gross examination was performed for evidence of oviduct hydrosalpinx or any other related abnormalities of oviducts. The severity of oviduct hydrosalpinx was scored based on the following criteria: no hydrosalpinx (0), hydrosalpinx detectable only under microscopic examination (1), hydrosalpinx clearly visible with naked eyes but the size was smaller than the ovary on the same side (2), equal to the ovary on the same side (3) or larger than the ovary on the same side (4). The excised tissues, after photographing, were fixed in 10% neutral formalin, embedded in paraffin and serially sectioned longitudinally (with 5 μ m/each section). Efforts were made to include cervix, both uterine horns and oviducts as well as luminal structures of each tissue in each section. The sections were stained with hematoxylin and eosin (H&E) as described elsewhere [5]. The H&E stained sections were assessed by a pathologist blinded to mouse treatment and scored for severity of inflammation and pathologies based on the modified schemes established previously [5, 14]. The oviducts were scored for both luminal dilation (0, no significant dilation; 1, mild dilation of a single cross section; 2, one to three dilated cross sections; 3, more than three dilated cross sections; and 4, confluent pronounced dilation) and inflammatory cell infiltration (0, no significant infiltration; 1, infiltration at a single focus; 2, infiltration at two to four foci; 3, infiltration at more than four foci; and 4, confluent infiltration). Scores assigned to individual mice were calculated into means $\pm$ standard errors for each group of animals.

2.4. Immunofluorescence assay

HeLa cells grown on coverslips and infected with chlamydia were fixed and permeabilized for immunostaining as described previously [15, 16]. Hoechst dye (blue, Sigma) was used to visualize nuclear DNA. For titrating IFUs from swab samples, a mouse anti-chlamydial LPS monoclonal antibody (mAb, clone# MB5H9, unpublished data) plus a goat anti-mouse IgG conjugated with Cy3 (red; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were used to visualize chlamydial inclusions. For titrating mouse antibodies, antisera collected on day 60 after inoculations were serially diluted. The diluted antiserum samples were reacted with *C. muridarum*-infected HeLa cells as antigens. The primary mouse antibody binding was visualized with a goat anti-mouse IgG (for total IgG level) or goat anti-mouse IgG1 or IgG2a (for isotyping) conjugated with Cy3 (Jackson ImmunoResearch). The immunofluorescence-labeled samples were observed under an Olympus AX-70 fluorescence microscope (Olympus, Melville, NY). The highest dilution of a given antiserum that remained positively reactive with *C. muridarum* antigens was defined as the titer of that antiserum. The final antibody titers were expressed as Log10 dilution.

2.5. *C. muridarum* fusion protein ELISA

To map the antigen specificities of the mouse antibodies, we reacted each mouse serum with each of the 257 *C. muridarum* proteins fused to the C-terminus of glutathione-S-transferase in a fusion protein ELISA as described previously [17, 18]. Briefly, bacterial lysates containing the GST fusion proteins were directly added to the 96 well microplates pre-coated with glutathione (Pierce, Rockford, IL) to allow GST to interact with glutathione. After washing to remove excess fusion proteins and blocking with 2.5% nonfat milk (in phosphate-buffered solution), individual mouse serum samples after appropriate dilutions were applied to the microplates. The serum antibody binding to antigens was detected with a goat anti-mouse IgG conjugated with HRP (Jackson ImmunoResearch Laboratories) in combination with the soluble substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; Sigma) and quantitated by reading the absorbance (OD) at 405 nm using a microplate reader (Molecular Devices Corporation, Sunnyvale, CA).

A reaction between a given fusion protein and an antiserum with an OD value equal to or greater than the mean plus 2 standard deviations (compared among all 257 antigens) was defined as positive. Antigens that positively reacted with 50% or more antisera from a given group of mice were considered immunodominant antigens.

2.6. In vitro restimulation of spleen cells for cytokine production and cytokine ELISA

The spleen cells were stimulated and cytokines were measured as described previously [19]. Briefly, the spleen cells harvested on day 60 after intravaginal or intrabursal inoculations were restimulated with live *C. muridarum* organisms for 72h. The supernatants were assayed for mouse cytokines IFN γ , IL-17 and IL-5 using ELISA kits (IFN γ , cat# DY485; IL-17, cat# M1700; IL-5, cat# DY405; R&D Systems, Inc, Minneapolis, MN). The cytokine concentrations were calculated based on absorbance values, cytokine standards and sample dilution factors.

2.7. Statistical analyses

Wilcoxon Rank Sum was used to compare the live organism shedding time courses while Kruskal Wallis was used to analyze nonparametric quantitative data including Log10 IFUs and OD values. Student t-Test was used to analyze cytokine concentration and IgG2a versus IgG1 ratio data. The semi-quantitative pathology scores were analyzed using Mann-Whitney Rank Sum Test. Fisher's Exact was used to analyze category data including the % of mouse

antisera that positively bound to a given antigen. The Log-Rank Test was used to compare the time course curves of mice that remained positive for shedding live organisms.

3. Results

3.1. Neither intrabursal nor intravaginal inoculation with *C. muridarum* is able to induce significant hydrosalpinx in DBA/1j mice

We previously showed that intravaginal inoculation with *C. muridarum* failed to induce hydrosalpinx in DBA/1j mice [10]. In the current study, we further tested whether intrabursal inoculation of live *C. muridarum* organisms directly into the oviduct was able to induce hydrosalpinx (Fig. 1). To our surprise, the intrabursal inoculation failed to induce any significant hydrosalpinx. The live *C. muridarum* organism shedding from the lower genital tract was similar between mice inoculated intrabursally or intravaginally. Although the intrabursally infected mice started with very low levels of live organism shedding on day 3, both groups of mice shared similar shedding time courses by day 14 (Fig. 1A). When the two groups of infected mice were sacrificed on day 60, only minimum levels of hydrosalpinx were detected in either group of mice. There were no significant differences in either the incidence or severity of hydrosalpinx between the two groups (1B). The genital tissues were further sectioned and stained with H&E for microscopic observation of inflammatory pathology in the oviduct (1C). Again, there was no significant difference in either inflammatory score or dilation score between mice infected intravaginally or intrabursally (1D).

3.2. Intravaginal inoculation is more effective than intrabursal inoculation in inducing protective immunity against intracervical challenge infection in DBA/1j mice

We next compared the intravaginal versus intrabursal inoculations for their ability to induce protection against an intracervical challenge infection (Fig. 2). DBA/1j mice intravaginally inoculated with SPG or intranasally with *C. muridarum* were used as negative and positive immunization control groups respectively. All 3 *C. muridarum*-inoculated or immunized groups displayed significantly lower levels of live organism shedding or more rapid clearance of live organisms compared to the negative immunization control SPG group. Careful comparison between intravaginally and intrabursally infected groups revealed that mice infected intravaginally developed more robust immunity against the intracervical challenge infection. Live organism sheddings from vaginal swabs were only positive on day 3 after the challenge infection in the intravaginally infected mice while the live organism shedding persisted in the intrabursally immunized mice for up to 21 days (Fig. 2A, $P =$ or <0.05). When mice were sacrificed 60 days after the intracervical challenge infection for observing oviduct gross pathology (Fig. 2B), no hydrosalpinx was detected in any of the intravaginally infected mice while 43% of the intrabursally infected mice developed some level of hydrosalpinx induced by intracervical challenge infection.

3.3. Characterization of immune responses in DBA/1j mice infected with *C. muridarum* via either intravaginal or intrabursal inoculations

We compared both cellular and humoral immune responses between mice infected via intravaginal or intrabursal inoculations. While both groups of mice developed T cell responses dominated by high IFN γ and IL-17 (Fig. 3), IL-5 was not detectable (data not shown). However, when the *C. muridarum*-specific IgG antibodies were isotyped, the ratios of IgG2a versus IgG1 were significantly higher in the intravaginally infected mice than the mice infected intrabursally (Fig. 4). We then further analyzed the antigen specificities of the IgG antibodies between the two groups of mice by reacting each mouse antiserum with each of the 257 GST-*C. muridarum* fusion protein antigens (Fig. 5). Sera from mice inoculated with SPG were used as negative controls. A reaction between a given antigen and a given

mouse antiserum with an OD value equal to or greater than the mean plus 2 standard deviations was considered positive. The antigens that positively reacted with 50% or more antisera from a given group of mice were considered immunodominant (Table 1). A total of 28 (out of 257) *C. muridarum* antigens were immunodominant in DBA/1j mice. When we compared both the antigen-antibody reaction intensity (OD values) and frequency (number of positive antisera for a given antigen) between the two groups of mice (Table 1), we found that of the 28 immunodominant antigens, 14 were commonly recognized by both groups while 3 (ribosomal protein S1, glycogen synthetase A or GlgA and GlgB) preferentially recognized by the intravaginally infected and 11 (including 4 hypothetical proteins TC0035, TC0912, TC0268 & TC0868 and 3 outer membrane proteins MOMP, OmcB and 15 kDa CRP) by the intrabursally infected groups.

4. Discussion

Just as not every woman sexually transmitted with *C. trachomatis* develops tubal factor infertility, not all inbred strains of mice intravaginally infected with *C. muridarum* develop hydrosalpinx/tubal infertility. We have recently found that DBA/1j mice fail to develop significant hydrosalpinx upon intravaginal infection [10] although many other strains of mice including Balb/c, C57BL/6j, SJL and C3H/HeJ or C3H/HeN are able to do so [5, 6, 10, 14, 20]. However, the DBA/1j mouse resistance to hydrosalpinx can be effectively overcome by intracervical inoculation [10]. Although it is ethically impossible to reproduce this finding in humans, it suggests that in some women, as in DBA/1j mice, the intravaginal chlamydial organisms may fail to ascend effectively. In the current study, we further characterized *C. muridarum* infection in DBA/1j mice. First, we found that intrabursal inoculation, by directly inoculating live chlamydial organisms into the oviduct, failed to induce significant hydrosalpinx. Second, intrabursal inoculation is not as effective as intravaginal inoculation in inducing protective immunity against intracervical challenge infection. Third, although both the intrabursal and intravaginal inoculations induced similar overall immune responses, mice immunized via each route did recognize unique antigens. These observations will not only facilitate our understanding of chlamydial pathogenesis but have also revealed that intravaginal immunization in combination with intracervical challenge infection in DBA/1j mice can be a useful model for analyzing the impact of the vaginal environment, such as the relationship between microbiome/metabolome and hydrosalpinx, and the efficacy of vaccines administered intravaginally.

Why was intrabursal inoculation unable to induce hydrosalpinx in DBA/1j mice? In strains of mice susceptible to hydrosalpinx upon intravaginal infection, intrabursal infection induced infertility at similar rates as intravaginal infection [9, 21]. The only major advantage of intrabursal inoculation was that there was no need to inject progesterone prior to the inoculation. It appears that *C. muridarum* organisms introduced into the mouse genital tract via either intravaginal or intrabursal inoculations display similar ability to cause tubal dysfunction. The lack of enhanced pathogenicity via intrabursal inoculation might be due to the lack of direct amplification of the intrabursally introduced organisms in the oviduct epithelial cells. The organisms inoculated into the oviduct may have to descend to the uterine epithelial cells for amplification. This hypothesis is supported by the live organism shedding time course observed in the intrabursally inoculated mice (Fig. 1). The shedding was very minimal at the initial stage and gradually reached the same level as in the intravaginally inoculated mice, suggesting that the organisms inoculated into the oviduct may be moved into uterine mucosa via an undefined mechanism. In this case, it may take time for the organisms to multiply in the uterine epithelial cells. Just as the intravaginally inoculated organisms are not able to effectively ascend to the oviduct [10], the intrabursally inoculated organisms may not only fail to directly replicate efficiently in the oviduct but also be unable to reproduce massive amounts of progeny in the uterine epithelial cells for

ascending back to the oviduct. Intracervical inoculation by directly delivering live organisms into uterine epithelial cells induced hydrosalpinx [10]. This observation suggests that DBA/1j mice are genetically capable of developing hydrosalpinx-causing inflammatory responses. We propose that lack of hydrosalpinx in the intravaginally or intrabursally infected DBA/1j mice is probably due to insufficient progeny live organism invasion into the oviduct tissue.

When immune responses were compared between intravaginally and intrabursally inoculated mice, intravaginal inoculation seemed to induce stronger protective responses against an intracervical challenge infection. Both inoculations induced robust cellular and humoral immune responses, and the overall phenotypes of the responses were similar between the two groups (Fig. 3 & 4). The main differences are the ratios of IgG2a versus IgG1 and preferentially recognized antigens by each group. The intravaginally inoculated mice displayed a significantly higher ratio of IgG2a versus IgG1, suggesting that this group of mice were more Th1-biased. A total of 28 *C. muridarum* antigens were recognized by 50% or more mice of either or both groups, and these antigens were defined as immunodominant antigens. Of the 28 antigens, 14 were commonly recognized by both groups. These antigens are hypothetical proteins, membrane proteins and enzymes. Importantly, the intravaginally inoculated mice preferentially recognized 3 antigens (ribosomal protein S1 and glycogen synthetase A and B or GlgA and GlgB) while the intrabursally immunized mice recognized 11 antigens (hypothetical proteins TC0035, TC0912, TC0268 & TC868, the outer membrane proteins 15 kDa CRP, OmcB, MOMP, ABC transporter, Mip, Protease IV and GroEL). GlgA is now known to be secreted into host cell cytosol [22]. We have previously shown that antibody production to secreted proteins is dependent on live organism infection [23] and antibodies against GlgA are produced in women urogenitally infected with *C. trachomatis* [22]. Thus, the preferential recognition of GlgA by antibodies from intravaginally inoculated mice suggests that the intravaginally inoculated *C. muridarum* organisms produce more GlgA or replicate better than the intrabursally inoculated *C. muridarum* organisms. This hypothesis is consistent with the finding that intravaginal inoculation induced stronger protective immunity. The observation that intravaginally infected mice also preferentially recognized GlgB that is functionally related to GlgA suggests that there may be active glycogen synthesis during *C. muridarum* intravaginal infection. In contrast, the intrabursally inoculated mice preferentially recognized 11 antigens, suggesting that these 11 proteins are highly expressed or highly immunogenic when *C. muridarum* organisms were directly introduced into the oviduct. Interestingly, among the 11 antigens are chlamydial organism outer membrane proteins MOMP (major outer membrane protein), OmcB (outer membrane complex B) and the 15 kDa cysteine-rich protein as well as GroEL, suggesting that *C. muridarum* organisms are stressed and may only have a limited replication capacity in the oviduct epithelia. These results together have demonstrated the flexibility of chlamydial protein expression during *in vivo* infection, which may partially explain the differences in host responses to chlamydial infection via different routes.

Since a single intravaginal infection with *C. muridarum* in most other mouse strains can result in significant hydrosalpinx, it has been difficult to study the impact of repeated intravaginal infections or intravaginal immunization with live organisms on hydrosalpinx. The DBA/1j mouse model has provided a new tool for investigating the mechanisms on how intravaginal infection/immunization or alteration of intravaginal microbiome may affect the incidence and severity of hydrosalpinx induced by intracervical infection.

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Highlights

Intracervical inoculation with *Chlamydia muridarum* induced hydrosalpinx in DBA/1j mice, but intravaginal or intrabursal inoculation failed to do so. Mice immunized intravaginally developed stronger protective immunity against intracervical challenge infection than mice immunized via intrabursal inoculation. The robust protective immunity correlated with higher ratio of IgG2a versus IgG1 and unique antigens recognized by antibodies in mice immunized intravaginally. Thus, intravaginal immunization, in combination with intracervical challenge infection in DBA/1j mice, can be a useful model for understanding mechanisms of chlamydial pathogenicity and protective immunity.

Fig. 1. Comparison of infection time course and oviduct pathology between DBA/1j mice infected with *Chlamydia muridarum* via intrabursal versus intravaginal inoculations

(A) Female DBA/1j mice inoculated with *C. muridarum* either intravaginally (ivag, filled triangle, n=13) or intrabursally (ib, filled circle, n=14) were swabbed on different days after inoculation (as shown along X-axis) for monitoring live organism shedding from the lower genital tract. The recovered live organism titers expressed as Log10 inclusion forming units (IFUs) per swab (panel a) and the % of mice remained positive for shedding live organisms (b) were shown along the Y-axis. Although the intrabursally infected mice started with low levels of live organism shedding on day 3, both groups of mice shared similar shedding time courses by day 14. (B) Portions of the infected mice described above were sacrificed on day 60 for observing hydrosalpinx (n=6 for ivag while n=7 for ib). Only minimum levels of hydrosalpinx were detected in both groups of mice (red arrows pointing to the mild hydrosalpinx in each group). There were no significant differences in hydrosalpinx between the two groups in terms of either the incidence or severity of hydrosalpinx. (C) The same tissues were also sectioned and stained with H&E for microscopic observation of inflammatory pathology in the oviduct under magnification of either 10x (panels a & b) or 40x (a1 & b1) objective lens. (D) The oviduct pathology was semi-quantitatively scored in terms of both inflammatory infiltration (left) and luminal dilation (right). Note that there was no significant difference in either inflammatory score or dilation score between mice infected intravaginally or intrabursally.

Fig. 2. Induction of protective immunity against intracervical challenge infection via intravaginal or intrabursal immunization

(A) DBA/1j mice inoculated intravaginally (ivag, filled triangle, n=7) or intrabursally (ib, filled circle, n=7) as described in Fig.1 legend were challenged intracervically (ic) with *C. muridarum* 60 days after the inoculation. Mice intravaginally inoculated with SPG [SPG (-), filled diamond, n=5] or intranasally [in(+), filled square, n=5] with *C. muridarum* were used as negative and positive immunization groups respectively. On various days after ic challenge infection as shown along the X-axis, mice were swabbed for monitoring live organism shedding. The results were expressed as Log10 IFUs per swab (panel a) and % of mice remaining positive for shedding live organisms (b). Note that all 3 *C. muridarum*-inoculated groups displayed significantly lower levels of live organism shedding or less numbers of mice shedding live organisms compared to the SPG group. Furthermore, ivag infected mice reduced live organism shedding more rapidly than ib inoculated mice. (B) All 4 groups of mice were sacrificed 60 days after the ic challenge infection for observing oviduct gross pathology hydrosalpinx. Note that both intranasally and intravaginally immunized mice were 100% protected from developing any hydrosalpinx while hydrosalpinx was detected in the intrabursally infected mice.

Fig. 3. T cell cytokine profiles in DBA/1j mice inoculated with *C. muridarum* intravaginally or intrabursally

Spleen cells harvested from DBA/1j mice inoculated with live *C. muridarum* either intravaginally (ivag, n=6) or intrabursally (ib, n=7) as described in Fig 1 legend were stimulated with (solid bar) or without (open bar) live *C. muridarum* EB organisms in cell culture for 72h. The supernatants were collected for detecting IFN γ (a), IL-17 (b) or IL-5 (data not shown). Note that high levels of IFN γ and IL-17 but no IL-5 were detected in all or any mice regardless of the inoculation/immunization groups.

Fig. 4. *Chlamydia muridarum*-specific antibody titers and isotypes in DBA/1j mice inoculated with *C. muridarum* intravaginally or intrabursally

Sera collected from DBA/1j mice inoculated with live *C. muridarum* either intravaginally (ivag, n=6) or intrabursally (ib, n=7) as described in Fig 1 legend were reacted with *C. muridarum*-infected HeLa cells, and the total IgG and isotypes IgG2a and IgG1 were titrated. The titers were expressed as Log10 dilution as shown along the Y-axis (a). The ratios of IgG2a versus IgG1 were further calculated (b). Note that although both iv and ib mouse groups developed similar high levels of anti-*C. muridarum* antibodies, the ratio of IgG2a versus IgG1 is significantly higher in intravaginally than intrabursally immunized mice.

Fig. 5. Mapping antigen specificities of antibodies from DBA/1j mice inoculated with *C. muridarum* intravaginally or intrabursally

Sera collected from DBA/1j mice inoculated with live *C. muridarum* either intravaginally (panels a & b, n=6) or intrabursally (c & d, n=7) as described in Fig. 1 legend were reacted with each of 257 GST-*C. muridarum* fusion proteins as shown along the X-axis. Both the reactivity intensity (expressed as OD values; a & c) and frequency (expressed as number of mouse sera positively recognized a given antigen; b & d) are shown along the Y-axis. Sera from mice mock immunized with SPG were used as negative control (e, n=5). Note that a positive antigen was defined as the antigen that reacted with a given mouse antiserum with an OD value equal to or greater than the mean plus 2 standard deviations when all 257 antigens were considered. The antigens that were positively reacted with 50% or more antisera from a given group of mice were considered immunodominant antigens.

Table 1

Immunodominant *Chlamydia muridarum* Proteins in DBA/1j

Binding Preference	ORF	Definition	Binding OD (Mean ± SD)		P value (Kruskal Wallis)	Binding Frequency (%)		P value (Fisher Exact)
			ivag	ib		ivag	ib	
14 antigens dominantly recognized by both ivag & ib mice	TC0177	Hypothetical protein	2.35±0.66	2.54±0.62	0.628	100%	100%	1
	TC0248	Hypothetical protein	1.17±0.41	1.61±0.43	0.101	100%	100%	1
	TC0816	Hypothetical protein	1.28±0.71	0.96±0.75	0.445	100%	100%	1
	TC0067	Hypothetical protein	0.17±0.16	0.37±0.24	0.073	67%	86%	0.416
	TC0117	Hypothetical protein	0.30±0.35	0.53±0.41	0.234	50%	86%	0.164
	TC0392	Hypothetical protein	0.24±0.25	0.68±0.50	0.101	50%	71%	0.429
	TC0708	Hypothetical protein	0.34±0.50	0.80±0.78	0.181	50%	71%	0.429
	TC0166	Hypothetical protein	0.48±0.39	0.13±0.24	0.181	67%	29%	0.17
	TC0279	Hypothetical protein	0.41±0.52	0.68±0.72	0.181	33%	86%	0.053
	TC0396	IncA	1.83±0.59	2.03±0.63	0.445	100%	100%	1
	TC0439	Adherence factor	0.58±0.75	0.40±0.69	0.366	67%	29%	0.17
	TC0152	mutT/Nudix family pr.	1.05±0.78	0.60±0.46	0.234	100%	86%	0.335
	TC0229	FtsH, putative	0.96±1.13	1.75±0.87	0.234	83%	100%	0.261
	TC0480	3-deoxy-D-manno-2-octulosonic acid transferase	0.37±0.23	0.50±0.25	0.366	67%	86%	0.416
3 preferred by ivag	TC0373	Ribosomal protein S1	1.10±1.06	0.17±0.14	0.022	83%	29%	0.048
	TC0181	GlgA	2.21±1.37	0.14±0.32	0.002	83%	14%	0.013
	TC0257	GlgB	1.79±1.07	0.74±0.60	0.073	100%	71%	0.155
11 preferred by ib	TC0035	Hypothetical protein	0.25±0.19	0.96±0.60	0.008	50%	100%	0.033
	TC0912	Hypothetical protein	0.30±0.15	1.14±0.28	0.001	50%	100%	0.033
	TC0268	Hypothetical protein	0.47±0.38	1.14±0.43	0.035	83%	100%	0.261
	TC0868	Hypothetical protein	0.16±0.14	0.79±0.60	0.014	50%	86%	0.164
	TC0726	15 kDa CRP	2.65±0.29	3.16±0.35	0.035	100%	100%	1
	TC0727	OmcB	2.25±0.55	2.90±0.18	0.008	100%	100%	1
	TC0052	MOMP	1.56±0.76	2.61±0.35	0.005	100%	100%	1

Binding Preference	ORF	Definition	Binding OD (Mean $\pm$ SD)		P value (Kruskal Wallis)	Binding Frequency (%)		P value (Fisher Exact)
			ivag	ib		ivag	ib	
	TC0660	ABC transporter	1.95 $\pm$ 0.64	3.10 $\pm$ 0.27	0.005	100%	100%	1
	TC0828	Mip	1.35 $\pm$ 0.67	2.49 $\pm$ 0.33	0.008	100%	100%	1
	TC0781	Protease IV, putative	0.28 $\pm$ 0.16	0.92 $\pm$ 0.36	0.005	83%	100%	0.261
	TC0386	GroEL	0.21 $\pm$ 0.23	0.74 $\pm$ 0.38	0.014	50%	86%	0.164