A comparison of the effects of a chlamydial vaccine administered during or after a *C. muridarum* urogenital infection of female mice.

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Running title

Effects of timing of chlamydial vaccination

Introduction

There are approximately 92 million new chlamydial infections of the genital tract in humans diagnosed each year [1], costing health care systems billions of dollars in treatment not only of acute infections, but also of associated inflammatory sequelae, such as pelvic inflammatory disease (PID) and ectopic pregnancy [2]. These numbers are increasing at a steady rate [3] and, due to the asymptomatic nature of infections, the incidence may be underestimated and the costs of treatment therefore higher. Over the previous few decades there has been a large amount of research into the development of an efficacious vaccine against genital tract chlamydial infections [4]. The majority of this research has focused on females, due to the high rate of development of associated diseases, including PID, which can lead to ectopic pregnancy and infertility [5]. In light of the increasing infection rates that have occurred despite the availability of antibiotics, and the asymptomatic nature of chlamydial infections, it is imperative that an efficacious vaccine that protects against infection and associated pathology be developed.

It is widely accepted that a protective chlamydial vaccine needs to induce a strong Th1 immune response, causing an increase in IFN-γ secreting $CD4^+$ T cell infiltration to the infection site [6], and induce the secretion of neutralizing antibodies at the mucosal surface [7]. However, there is the chance that an overstimulation of the immune system with vaccination could lead to the development of enhanced pathology. For example, the increased production of TNF-α has been shown to aid in clearance of a genital infection [8, 9], but it is also a pro-inflammatory cytokine that can stimulate the production of other cytokines. This enhancement of cytokine

production can contribute to immune-mediated damage to the reproductive tract [10-12]. Therefore, any successful vaccine must contain both antigens and adjuvants that induce a balance between providing protective immunity and not contributing to immune-associated disease pathogenesis.

Major issues relating to chlamydial vaccine development, which have not been examined to date, are the effect of vaccination on an individual that has previously been exposed to an infection, and how re-exposure of the individual to a genital infection after vaccination may affect the final outcomes. Very early trachoma vaccine trials, that used whole killed *Chlamydia* as the vaccine*,* examined the effects of immunization on blind individuals who had had a previous trachoma infection [13, 14]. These individuals were then given a second chlamydial ocular infection, and it was found that those that were immunized developed more severe pathology, compared to those that were mock immunized. Very similar studies have been performed in monkeys revealing the same phenomenon [15, 16]. It was suggested that the enhancement of pathology in the monkeys was due to the high homology between chlamydial heat shock protein – 60 (HSP-60) and self HSP-60, contributing to autoimmunity [16]. Due to the advancement of molecular technologies, individual antigens can now be identified and screened, thereby avoiding possible molecular mimicry. Because the rates of genital tract chlamydial infections are steadily rising, and therefore the rates of previous exposure, research into this aspect of vaccine development is essential.

The effect of vaccination of individuals who have an active chlamydial genital tract infection is another important aspect of chlamydial vaccine studies that has not yet

been examined in any way. Due to the asymptomatic nature of chlamydial genital tract infections, with approximately 70% of infected women showing no symptoms [17], it is imperative that this aspect, of vaccination during infection be examined. While it is not expected, it is possible that vaccination during an acute infection may affect the course and degree of infection in the genital tract. It is unknown whether vaccination will boost the already active immune system and enhance clearance, or if any enhancement of the immune response will further exacerbate pathology development. Further stimulation of immune cells through immunization may increase pro-inflammatory cytokine production and further increase cellular infiltrate into the genital tract, contributing to the development of long term sequelae in women.

The current study utilized the already developed vaccine candidates, the major outer membrane protein (MOMP) [18, 19] and ribonucleotide reductase small chain protein sub-unit B (NrdB) [20] of *C. muridarum*, administered intranasally, to examine the effects of timing of vaccination in a murine model of chlamydial genital tract infection. Firstly, we aimed to examine whether immunization during an acute genital infection, or after clearance of a genital infection, enhanced immune responses above that induced by an infection alone. Secondly, we aimed to determine if the timing of vaccination enhanced or reduced the development of pathology in the reproductive tract of mice immunized with our vaccines. Finally, we aimed to determine whether immunization during a genital infection had any therapeutic effect, and also whether it provided extra protection against a second genital tract chlamydial infection.

Materials and Methods

Chlamydia strain

Chlamydia muridarum (Weiss; ATCC VR-123, Virginia, USA), formerly the mouse pneumonitis biovar of *C. trachomatis* (MoPn), was grown by inoculation of McCoy cell (ATCC CRL-1696, Virginia, USA) monolayers in Dulbecco's minimal essential medium supplemented with 5% fetal calf serum, 2mM L-glutamine, 100µg/mL Streptomycin sulfate, 2µg/mL Gentamycin and 20mM HEPES. Elementary bodies were purified using a discontinuous Renografin gradient as previously described [21].

Protein production/purification

Major outer membrane protein (MOMP)

The transformed *Escherichia coli* (DH5α{pMMM3}) expressing the pMAL-c2 vector encoding recombinant MBP-MOMP fusion protein was a generous gift from Harlan Caldwell (Rocky Mountain Labs, Hamilton**,** MT, USA). MBP-MOMP was produced as previously described [18].

Ribonucleotide reductase small chain protein sub-unit B (NrdB)

Transformed *E. coli* (BL21 DE3) expressing the pCOLD vector (TaKaRa, Shiga, Japan) encoding recombinant His-tagged NrdB was produced and purified. Clonal *E. coli* colonies were grown overnight at 37ºC in 5mL LB broth containing ampicillin. This was transferred to 500ml of LB broth containing ampicillin and cultured at 37ºC and 225rpm until an OD measurement of 0.4-0.5 was obtained at 600nm. Cultures were chilled on ice for 30mins. Protein production was induced by the addition of 0.5mM IPTG (Sigma-Aldrich, North Ryde, NSW, Australia), final concentration, and

cultures were incubated overnight at 15°C and 225rpm. Cells were collected via centrifugation at 10,000 x g for 10 minutes at 4°C. Pellets were resuspended in 50mL PBS, 0.15g Lysozyme (Sigma-Aldrich) was added and cells incubated at 37°C for 1 hour. Bacterial protease cocktail inhibitor (Sigma-Aldrich) was added according to the manufacturer's instructions and 1mM PMSF (Sigma-Aldrich), final concentration, was added. Cells were aliquoted to 10mL and sonicated in 15 second cycles a total of 8 times, after which time they were spun at 4,000 x g, 4°C for 10 minutes. Supernatants were then spun at 10,000 x g for 10 minutes at 4°C, and supernatants frozen at -20°C until purification.

Purification of NrdB was performed using the batch/gravity-flow column purification as described by the manufacturers (Talon Resin, BD Biosciences, North Ryde, NSW, Australia). Eluted protein fractions were analyzed via SDS-page. Fractions containing protein were dialyzed using cellulose dialysis membrane with an 8kDa cut-off (Sigma-Aldrich) in PBS, and then concentrated using Amplicon concentrators (Millipore, North Ryde, NSW, Australia) with a cut-off of 10kDa.

Mice

Female BALB/c mice, 6-8 weeks of age, were obtained from The Animal Resource Centre, Perth (Australia), and housed in an accredited laboratory animal care facility under specific-pathogen free conditions. Animals received food and water *ad libitum*. All procedures were approved by the Queensland University of Technology Animal Research Ethics Committees. Five animals were used in each group and each experiment was repeated twice.

Infection and monitoring

Mice were given 2.5mg of medroxyprogesterone acetate (Depo-Provera, Pfizer, West Ryde, NSW, Australia) subcutaneously, seven days prior to infection. The mice were anaesthetised intraperitoneally using ketamine (90 mg/kg; Parnell Laboratory, Alexandria, NSW, Australia) and xylazine hydrochloride (10 mg/kg; Bayer, Pymble, NSW, Australia) and infected intra-vaginally with 20µl of sucrose-phosphate-glutamate (SPG) containing 5 x 10^4 inclusion forming units (ifu) of *C. muridarum*.

Immunization

Animals were lightly anaesthetized with 4% isoflurane. Appropriate, equal volumes of immunization solution were placed on each nare for animals to inhale. Immunization solutions consisted of 5µg cholera toxin (CT; List Biological Laboratories, Campbell, CA, USA), 10µg CpG-ODN (sequence: 5'-TCCATGACGTTCCTGACGTT -3'; Sigma-Aldrich), and 50µg of either MOMP alone or 50µg of MOMP and 50µg NrdB in combination. All immunization solutions were made up to equal volumes using sterile PBS. Mice were immunized naïve, during an acute genital chlamydial infection, or after clearance of a genital chlamydial infection in accordance with figure 1.

Detection of *C. muridarum* infection

Infection was monitored by collecting cervico-vaginal swabs (Copan, Murrieta, CA, USA) every three days post infection. Swabs were placed in tubes containing 500µl SPG and glass beads and were stored at -80°C. To monitor infection levels, swabs were cultured as described elsewhere [22]. The chlamydial inclusions were visualised by staining with rabbit anti-*C. trachomatis* antibody (Pierce/Progen,

Richlands, Australia) and Immunopure ABC/DAB Staining Kit (Pierce/Progen, Richlands, Australia), as described elsewhere [18].

Sample collection

At the termination of the experiments (Fig. 1), animals were euthanized using a lethal injection of sodium pentobarbitone. Blood was collected via cardiac puncture, centrifuged at 12,000 x g and serum was collected. Vaginal lavage was collected by flushing the vaginal vault with 40µL of sterile PBS and uterine lavage was collected by removing the horns from the animal and flushing with 100µL sterile PBS. All samples were stored at -20°C until testing.

T cell proliferation and cytokine assessment

Spleens were removed from animals and processed as described elsewhere [18]. Cells were plated into 96 well tissue culture plates at 5 x 10^5 cells/well. Stimulants were added to wells (2 µg/well MOMP, 2 µg/well NrdB or media) and cells were incubated for 4 days. Supernatants were collects and centrifuged at 500 x g for 10 mins to removed debris. Supernatants were assayed for IL-4, IL-10, IFN-γ and IL-12p(70) using Bio-Plex bead array system (Bio-rad, North Ryde, NSW, Australia) according to manufacturer's instructions.

ELISA's

Ninety six well plates (Interpath, Heidelberg West, VIC, Australia) were coated with 2µg/well of protein (MBP-MOMP or His-NrdB) and incubated at 4°C overnight. ELISA's were performed as described previously [18]. The antibodies used were anti-mouse IgG or anti-mouse IgA (Southern Biotechnology Associates, AL, USA) at

a 1/1000 dilution, and horseradish peroxidase (HRP)-conjugated streptavidin (Amersham Life Science, NJ, USA) at a 1/1000 dilution. The end point titre (EPT) was determined by the dilution at which the absorbance became equivalent to that of the EPT threshold. The EPT threshold was calculated as the average absorbance of the no sample control + 2 standard deviations.

Gross pathology

After euthanasia, the reproductive tracts were examined *in situ* for macroscopic changes. The degree of hydrosalpinx development was recorded and grouped as described previously [22]. Briefly, swelling of the oviduct between 2-3mm was scored 1, 3-5mm was scored 2 and >5mm was scored 3.

Histological assessment

Excised genital tract tissues were removed at euthanasia, fixed in 10% formaldehyde and embedded in paraffin wax. Five µm sections were cut, dewaxed and rehydrated through graded ethanol solutions to PBS. Haematoxylin and eosin staining was performed. Ten random fields (× 1000 magnification) of each tissue were counted ensuring to include both epithelium and sub-mucosa, with the observer blinded to the group being examined.

Statistics

All data presented are mean ± standard error of mean (SEM). All statistics were performed using GraphPad Prism version 5.00 (GraphPad Software, La Jolla, CA, USA). Significant differences for the ELISA, hydrosalpinx and inflammatory cell infiltrate was determined using two-way ANOVA, with Bonferroni's post test and

significance was set at $p<0.05$. Unpaired students t-tests were used to calculate significant differences in clearance, with significance set at $p<0.05$.

Results

The effects of the timing of vaccination on antibody production, hydrosalpinx formation and inflammatory cell infiltration were examined. In the case of those animals immunized during an acute infection, the therapeutic effect of the vaccination was investigated, as was the effect on a secondary infection. Animals were immunized intranasally (IN) with the adjuvants cholera toxin (CT) and CpG-ODN, and the antigens the major outer membrane protein (MOMP) or MOMP and ribonucleotide reductase small chain protein subunit B (NrdB) in combination (Fig. 1). These vaccine formulations have previously proven to induce T cell and antibody responses, and provide protection against genital infection [18-20].

The timing of vaccination alters the systemic but not local anti-chlamydial antibody production

Examination of MOMP and NrdB–specific systemic IgG in response to immunization of naïve mice and those animals immunized at various stages of a chlamydial infection revealed that the timing of vaccination had an effect on systemic antibody production. The infection alone groups served as time point controls, as they were infected, only once, at the same time as those that received any form of immunization, allowing any enhanced antibody production as a result of vaccination to be determined. The live immunization controls (LIC) were infected twice, with infections 10 weeks apart, and served as the positive control.

In terms of MOMP-specific serum IgG production (Fig. 2A) the greatest effects were seen in those animals that were immunized during infection. While IN immunization

of naïve mice with CT/CpG/MOMP produced a significant ($p<0.001$) amount of serum IgG compared to the LIC group (Fig. 2A), those immunized during infection with the same formulation produced approximately 3 fold more MOMP-specific IgG ($p<0.001$). Examination of systemic NrdB-specific IgG production (Fig. 2B) revealed that those mice receiving CT/CpG/MOMP/NrdB had similar levels of NrdB and MOMP-specific antibodies present. Immunization with the dual antigen formulation, during an infection, caused a significant increase ($p<0.001$) in NrdB-specific antibody production than when administered to naïve mice (Fig. 2B). NrdB antibodies were not detected in the animals that were infected only, or in the animals in the LIC group. Interestingly, there was no enhancement of MOMP or NrdB-specific IgG produced when animals were re-challenged with *C. muridarum* after infection and immunization (Fig 2A and 2B).

While immunization of mice with the various vaccine compositions produced MOMP–specific IgA at the vaginal mucosa (Fig. 3A), the timing of vaccination did not affect the levels of MOMP-specific IgA, as none were significantly greater than the infection only animals. Specifically, those animals immunized with CT/CpG/MOMP/NrdB after clearance of infection produced 2-fold less MOMP-specific IgA than those immunized naïve ($p<0.01$) or during an infection ($p<0.05$; Fig. 3A). Production of NrdB-specific IgA at the vaginal mucosa (Fig. 3B) was approximately 10-fold less compared to the levels of MOMP-specific IgA detected. There was a trend found, with those immunized with CT/CpG/MOMP/NrdB, during an infection, producing greater amounts of NrdB specific antibodies than those immunized naïve with the same formulation.

Examination of MOMP-specific IgA in uterine lavage (Fig. 4A) revealed that, while IgA production occurred, the timing of immunization did not significantly affect the levels of antibody being produced. Production of NrdB-specific IgA in the uterine horn (Fig. 4B) was most evident in those mice immunized after clearance of infection, with those immunized with CT/CpG/MOMP/NrdB producing the greatest amount; however this was not significantly greater than that detected in animals that were only infected.

Immunization leads to a balanced Th_1 and Th_2 T cell response.

The production of IL-4 and IL-10 were measured to determine the Th_2 response in animals and the production of IL-12p(70) and IFN-γ to determine the Th_1 response. In those animals that were immunized naïve (without infection) there were very low levels of all cytokines produced in response to MOMP or NrdB stimulation (Fig. 5A). In those animals receiving vaccination during an infection there was an overall balanced Th_1 verses Th_2 response with production of both IL-10 and IFN-γ in the CT/CpG/MOMP/NrdB group (Fig. 5B). The CT/CpG/MOMP group produced minimal cytokines in response to stimulation. In the mice immunized after clearance of an infection mice receiving either single or dual antigen vaccines produced IL-4, IL-10, IFN-γ and low levels of IL-12p(70) indicating both a Th_1 and Th_2 T cell immune response (Fig. 5C). Overall the addition of MOMP was the strongest stimulant, causing the majority of cytokine production.

Immunization has no therapeutic effect and does not provide enhanced protection against re-exposure

Mice were immunized either with a single or dual antigen vaccine naïve or during an acute *C. muridarum* vaginal infection. Comparison of those immunized naïve (Fig. 5A) and during an infection (Fig. 5B) with a single antigen vaccine demonstrates that there is no difference in the levels or course of infection. For those mice immunized with the dual vaccine formulation it can be seen that although those immunized naïve still have a very low level of infection at day 21 post infection (only one positive animal), this is not significantly different from those immunized during infection who clear infection at day 18. It can be seen in figure 6B that there were no significant differences detected between the mice that received only the adjuvants and those that received the complete vaccine formulations. There were also no significant differences observed in the rate of clearance of *Chlamydia* from the lower genital tract of mice either immunized with the complete vaccine formulations or immunized with adjuvants only.

The effects of re-exposure to infection after clearance of an infection and immunization were examined. Upon secondary genital infection with *C. muridarum* all animals in all groups became re-infected at very low levels, in comparison to the level of infection seen on primary exposure, including the animals receiving live immunizations (LIC; Fig. 6C). Interestingly, even though all animals had previously been exposed to a genital infection and immunized with our vaccine candidates, they still became infected upon secondary infection. However, the level of *C. muridarum* recovered from the lower genital tracts of animals receiving the secondary infection did not reach the challenge dose of 5×10^4 ifu and the course of infection was also reduced from 28 days to 9 days.

The timing of vaccination has no effect on the development of hydrosalpinx

The degree of hydrosalpinx formation in mice was measured. This revealed that there were no significant differences in the scores between any of the groups at any of the time-points examined (Fig. 7). There was, however, a trend showing less severe hydrosalpinx development in those mice immunized with the complete vaccine formulations after clearance of infection. This highlighted that the timing of vaccination did not enhance gross pathology development.

The timing of vaccination does not affect inflammatory cell infiltration into the upper reproductive tract

The inflammatory cell infiltrate into the uterine horns of animals was examined via H&E staining. There was a trend with animals immunized intranasally with the complete vaccine formulations after infection having greater neutrophil influx than control mice (Fig. 8A); however this was not above that of the infection control, suggesting the immunizations did not cause this infiltration. There were no significant differences observed in lymphocyte infiltration (Fig. 8B) between any of the groups immunized at the various stages of infection. Assessment of the reproductive tissues of those immunized after an infection and those re-exposed to an infection, revealed a slightly higher, but not significant, infiltration of plasma cells, than that seen in the infection control animals (Fig. 8C). Importantly, comparison of those animals immunized either during or after infection to those immunized before infection demonstrates that the timing of vaccination does not influence the inflammatory cell infiltration into the upper reproductive tract.

The inflammatory cell infiltrate into the oviducts of mice was examined, and it was found that there was no cellular infiltrate present (data not shown). However, due to the development of hydrosalpinx, the oviducts had lost their natural structure, including the loss of the ciliated epithelium. The oviduct of an uninfected mouse had normal plicae with a ciliated epithelium and secretory cells present. In comparison, those mice that were infected with *C. muridarum* had developed hydrosalpinx, which caused the destruction of ciliated epithelium and a total loss of structure within the oviduct, including loss of plicae and a flattened or denuded epithelium that is consistent with pressure atrophy.

Discussion

The number of *C. trachomatis* infections of the female reproductive tract has been steadily increasing over the last 10 years [23], and this current number could quite possibly be higher, due to the asymptomatic nature of infections. This highlights the need for the development of an efficacious vaccine that protects not only against infection, but also prevents the development of any pathological sequelae. Even with the considerable amount of research into the development of a *Chlamydia* vaccine, there has been no examination of the effects of the timing of vaccination, with regard to immunization after previous exposure or during an acute infection.

We have, for the first time, examined the effects of administration of a single and multi-subunit vaccine candidate on the subsequent immune responses of animals acutely or previously infected with *C. muridarum*. This study also investigated, for the first time, any effects the timing of immunization had on the development of infection-associated pathology, including hydrosalpinx formation and inflammatory cell infiltration into the upper genital tract. It was found that while the timing of vaccination affected the systemic production of antibodies, it had a minimal effect on the secretion of mucosal antibodies in the vagina and uterus of the mice. Of importance, it was found that the timing of immunizations did not significantly enhance the development of gross pathology or inflammatory cell infiltrate into the upper reproductive tracts of vaccinated mice. This is the first time that any chlamydial vaccine candidate has been used to examine the effects of immunization on those animals with a genital tract infection or those previously exposed to such an infection.

We observed that immunization during an acute infection or after clearance of one, boosted systemic MOMP-specific IgG antibody production, and to lesser extent NrdB-specific antibodies, however, this did not occur at the mucosal surfaces. The vaccine candidates used in this study have previously been shown to induce strong mucosal antibody responses [19, 20, Cunningham, unpublished data], however in our current study, vaginal and uterine lavage IgA antibodies detected were not above those of the infection time point controls, indicating that immunization did not enhance their secretion. NrdB is expressed by the reticulate bodies of *Chlamydia* during the intracellular stage of infection; therefore antibodies against NrdB would be expected to be found in animals that have had an infection. However, in this study animals that had been infected either once or twice did not produce systemic NrdB-specific IgG, the reason for this is unknown. A similar situation has been observed in varicella zoster virus (VZV) vaccine trials in seropositive adults, where vaccination was via inhalation [24]. Those individuals given live attenuated or heat killed vaccines had a > 2 fold increase in systemic antibodies, but had no significant increase in secretory IgA in the saliva [24]. Also, in Herpes simplex virus – 2 (HSV-2) vaccine trials, individuals who were seropositive for HSV-1 but seronegative for HSV-2, and those who were seronegative for both, were immunized against HSV-2 infection. It was found that the vaccine was only effective in those seronegative to both HSV-1 and 2 [25]. This is suggested to occur because immunity to the virus has been previously provided by infection with a different strain and administration of a vaccine does not enhance any response [25], and may also be the case seen in the results presented in this study.

In terms of the T cell production of cytokines, quite the opposite was seen, with increases in IL-10 and IFN-γ in those mice immunized during or after clearance of infection in comparison to those immunized naive. These increases are likely due to the fact that a longer time post infection has elapsed enabling the vaccination to boost the developed T cell memory response.

The inability of animals in our study to mount an enhanced immune response after vaccination, when either actively, or previously infected with *C. muridarum*, may be partly due to dendritic cell (DC) function. When animals become infected, immature DCs phagocytose chlamydial particles, process them, and present the chlamydial antigens through MHC molecules, thereby becoming mature DCs [26]. During the maturation process, upon exposure to *Chlamydia*, DCs also up-regulate their expression of surface receptors, including CD80, CD40 and CD86 [27]. However, it has recently been shown that DCs, isolated from BALB/c mice, infected with *C. muridarum* down-regulate both CD80 and CD86 surface receptors [28], which is able to subvert the normal function of these cells from normally inducing a Th1 response, to causing a Th2 response [28]. More importantly, once DCs become mature, they have been found to down-regulate MHC II synthesis, which prevents the formation of new MHC II peptide complexes in mature DCs [29]. Both a down-regulation of surface receptors due to infection and a down-regulation of MHC II complexes may contribute to the lack of enhanced immune response in the animals immunized either during or after infection in this study. This is likely due to the inability of DCs to process and present new/more antigens to B cells, thereby not enhancing the number of antigen specific B cells, and therefore restricting the levels of antibody production. *Chlamydia* infection is also able to inhibit IFN-γ-inducible MHC II

474 expression [30], possibly further reducing the host's ability to mount an effective
475 immune response.
476
477 In this study, it was found that the complete vaccine compositions had no therapeutic
478 effect. This was not surprising, as there are currently no therapeutic vaccines that
479 target the female reproductive tract approved for human use. However, there are
480 some in development, including those against human immunodeficiency virus (HIV)
481 and human papillomavirus (HPV). HPV studies in rabbits, using the cottontail rabbit
482 papillomavirus, and a HPV L1 virus-like particle (VLP) vaccine, have demonstrated
483 regression of established papillomas [31], and other studies using various delivery
484 systems and HPV antigens have shown a reduction in disease or regression of warts
485 [32]. Clinical trials of the human approved HPV vaccine, Gardasil, revealed that it
486 had no therapeutic effect on those who were already seropositive for HPV [33].
487 Since HIV was identified as the aetiologic agent for AIDS, there have been more
488 than 40 therapeutic vaccine candidates that have progressed to human clinical
489 testing, with little success [34]. The use of MOMP or MOMP and NrdB in
490 combination, administered intranasally, after clearance of a genital infection did not
491 provide protection above that induced by the infection alone. These antigens have
492 previously proved to provide partial protection against a genital infection in mice
493 immunized naive [18, 20]. However, this study highlights that a vaccine with antigens
494 that induce greater protective immunity and perhaps different adjuvants, would be
495 required to provide total protection against re-infection. At this point in time a vaccine
496 that provides total protection against a genital tract chlamydial infection does not
497 exist.
498

In this study the effects of the timing of vaccination on the development of upper reproductive tract pathology in mice was also examined. We have demonstrated that neither immunization during an active genital tract chlamydial infection, nor after clearance of a primary chlamydial genital infection, significantly enhanced hydrosalpinx development or inflammatory cell infiltration. However, while it did not enhance pathology, it also did not protect animals from further pathology upon re-infection of the genital tract. Those animals that were re-challenged with *C. muridarum* after having received an immunization after clearance of a primary infection still developed hydrosalpinx. Interestingly, we also observed that those animals that were immunized after infection had lower levels of hydrosalpinx development than the other groups examined. Hydrosalpinx occurs when the oviducts have become occluded from scar tissue development or cellular debris accumulation, causing clear, serous fluid to accumulate and dilate the oviduct [35]. It is also used as a marker of infertility in the mouse model [35, 36]. It has been suggested, that over time, hydrosalpinx is able to resolve in immunocompetent mice [37], which might have occurred here due to the extended time period of the experiment. Hydrosalpinx fluid has also been suggested to leak into the uterine cavity [38, 39], and may also explain some of the decrease in severity seen here.

Importantly, immunization with these particular sub-unit vaccines during an infection, or after clearance of a genital infection did not enhance inflammatory cell infiltration into the upper reproductive tract. It is highly possible, depending on the particular vaccine candidate and the type of immune response mounted, that an immunization during, or after an infection, could enhance pathology development [40]. Both antigens used here, administered intranasally with CT and CpG, have previously

been shown to cause the production of cytokines, including IFN-γ, TNF-α and IL-10 [18, 20, Cunningham, unpublished data]. The production of IFN-γ by $CD4^+$ T cells has proved to be essential in the clearance of *Chlamydia* from the genital tract. However, secretion of other pro-inflammatory cytokines, including TNF-α, has been suggested to be involved in triggering inflammation [17] and infection related pathologies such as salpingitis [41]. The induction of these other pro-inflammatory cytokines may increase the risk of further upper reproductive tract pathology enhancement. Previous trachoma vaccine trials used whole killed *Chlamydia* to immunize previously infected blind individuals [14]. It was found that those that had been previously exposed to infection developed a greater degree of pathology upon re-exposure, compared to those individuals who had not been previously infected [14]. Similar studies were performed in monkeys and similar results were obtained [15, 16]. This was thought to be linked to the presence of chlamydial HSP-60 and cross-reactivity with self HSP-60 and suggests that if chlamydial HSP-60 was to be used as an antigen this would enhance pathological sequelae development in the reproductive tract. This highlights the importance of examination of antigens under the conditions presented here.

Overall, we have demonstrated for the first time that the timing of vaccination does not enhance the development of infection-associated reproductive tract pathology, and have shown that these same vaccinations do not boost the local infection-induced antibody response in the female genital tract of mice. This does not mean that other sub-unit vaccines will not induce a greater memory response and cause pathological sequelae, but highlights the importance of trialing vaccines under various host infection states. Based on the studies presented here, if similar results

were obtained from human clinical trials, it would not be cost effective to vaccinate individuals who were seropositive for a current or previous *C. trachomatis* infection, as it would not provide enhanced protection. This is the first time that a sub-unit vaccine candidate has been used to examine the effects of immunization during an active, possibly asymptomatic genital infection, or after clearance of a previous genital infection. These are important factors that need to be examined during vaccine design due to the asymptomatic nature of infection and the increasing rate of infections.

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