Immunity against a Chlamydia infection and disease may be determined by a balance of IL-17 signaling

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Running title: Balance of IL-17 signaling may dictate protection

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Abstract

Most vaccines developed against *Chlamydia* using animal models provide partial protection against a genital tract infection. However, protection against the oviduct pathology associated with infertility is highly variable and often has no defining immunological correlate. When comparing two adjuvants (CTA1-DD and a combination of Cholera toxin plus CpG-oligodeoxynucleotide–CT/CpG) combined with the chlamydial major outer membrane protein (MOMP) antigen and delivered via the intranasal (IN), sublingual (SL) or transcutaneous (TC) routes, we identified two vaccine groups with contrasting outcomes following infection. SL immunization with MOMP/CTA1-DD induced a 70% reduction in the incidence of oviduct pathology, without significantly altering the course of infection. Conversely, IN immunization with MOMP/CT/CpG prevented an ascending infection, but not the oviduct pathology. This anomaly presented a unique opportunity to study the mechanisms by which vaccines can prevent oviduct pathology, other than by controlling the infection. The IL-17 signaling in the oviducts was found to associate with both the enhancement of immunity to infection and the development of oviduct pathology. This conflicting role of IL-17 may provide some explanation for the discordance in protection between infection and disease and suggests that controlling immunopathology, as opposed to the rapid eradication of the infection, may be essential for an effective human chlamydial vaccine that prevents infertility.

Keywords: adjuvant, *Chlamydia*, intranasal, sublingual, transcutaneous, vaccine.

Introduction

With over 250,000 new infections contracted daily worldwide [1], *Chlamydia trachomatis* is the most common cause of bacterial sexually transmitted infection. Infections can be easily treated with antibiotics, although many infections go untreated, as more than 70% of females show no signs or symptoms of an active chlamydial genital tract infection [2]. Pelvic inflammatory disease due to untreated ascending infection occurs in 10-40% of infected women [1] and infection of the oviducts/Fallopian tubes can cause tubal scarring and occlusion, ectopic pregnancy and infertility. As the burden of caring and treating patients with fertility complications costs in the order of $10 billion dollars each year in the US alone [3], a vaccine has the greatest potential to reduce both infection and disease prevalence [4].

Mathematical modeling has suggested that a vaccine capable of eliciting "sterilizing immunity" could eradicate *Chlamydia* from the human population within 20 years [5], but due to the nature of the pathogen and the physiology of the genital tract it infects the induction of sterilizing immunity against *Chlamydia* through vaccination is becoming increasingly improbable [6]. If sterilizing immunity is not possible, then the goal of a vaccine against *Chlamydia* should be to limit transmission and prevent infertility. However, decreasing the risk of transmission by reducing the amount and/or duration of chlamydial shedding during an infection, an outcome accomplished by most experimental vaccines, often fails to prevent disease [7]. Developing an understanding of the processes by which some vaccines either succeed or fail to prevent upper genital tract pathology is essential for the development of a vaccine that is effective against *Chlamydia*-induced infertility.

It is widely believed that a chlamydial vaccine will need to elicit mucosal immunity, which is vital to prevent infection by pathogens that enter via a mucosal epithelium [8]. Vaccines targeted to cutaneous/mucosal inductive sites like the skin (transcutaneous – TC), nasal (intranasal – IN) and buccal (sublingual – SL) mucosa are known to elicit mucosal immunity

more so than vaccines given by injection [9]. However, the success of a "needle-free" non-replicating vaccine is heavily reliant on a potent adjuvant to overcome the induction of mucosal tolerance without harmful side effects [10, 11]. The CTA1-DD adjuvant consists of the enzymatically active CTA1 subunit of Cholera toxin (CT), genetically linked to a dimer of an Ig-binding domain (DD) from the staphylococcal protein A [12]. CTA1-DD retains the adjuvanticity of the native holotoxin, but most importantly not its toxicity [12, 13], which makes CTA1-DD an excellent candidate for a chlamydial mucosal vaccine.

To develop a vaccine against *Chlamydia* that protects against both infection and upper genital tract pathology we compared two mucosal adjuvants, CTA1-DD [12] and a CT plus cytosine-phosphate-guanine-oligodeoxynucleotide (CT/CpG) combination [14]. These two adjuvants were combined separately with the recombinant chlamydial major outer membrane protein (MOMP) and delivered via the IN, SL or TC routes. Following a live intravaginal challenge with *Chlamydia muridarum*, the magnitude of the infection and the development of oviduct pathology were assessed. Vaccines found to confer a unique phenotype of protection against infection and/or oviduct pathology were subjected to a more comprehensive analysis to determine the immune responses required for immunity against infection and pathology.

Results

Protection against oviduct pathology following a live C. muridarum *vaginal challenge of vaccinated animals*

Following challenge with *C. muridarum*, we assessed the development of hydrosalpinx (Figure 1A), both its severity (oviduct diameter) (Figure 1B) and incidence (presence or absence) (Figure 1C), on day 49 post-infection (p.i). The unimmunized group developed severe pathology, with 100% of mice affected. The live infection control animals developed a similar severity and incidence of hydrosalpinx to the unimmunized group. Immunization with MOMP and CT/CpG via the SL and TC routes significantly reduced the severity ($P<0.05$) and incidence of hydrosalpinx ($P<0.05–0.01$) when compared to unimmunized controls. IN immunization with the same vaccine decreased the severity of hydrosalpinx, yet 100% of animals still displayed evidence of oviduct occlusion. SL immunization with MOMP and CTA1-DD offered the most significant protection against the severity ($P<0.01$) and incidence ($P<0.01$) of oviduct pathology, where only 20% of these vaccinated animals were affected compared to 100% in the unimmunized controls. IN immunization with MOMP and CTA1-DD resulted in a non-significant reduction in both the severity and incidence of hydrosalpinx. TC immunization with MOMP and CTA1-DD conferred no protection against pathology.

Protection against infection following a live C. muridarum *vaginal challenge of vaccinated animals*

To determine whether a decrease in the magnitude of the chlamydial infection was responsible for the observed reduction in oviduct pathology of vaccinated animals, we quantified the bacterial burden in the tissues of the genital tract at the peak of infection on day 6 p.i (Figure 2A) and the duration of vaginal shedding over the course of the infection (Figure 2B). In the unimmunized animals, the greatest quantity of *Chlamydia* was detected in the cervico-vagina, collected by the vaginal swab. Proportionally, the oviducts contained the

highest concentration of *Chlamydia* of all tissues in unimmunized animals, followed by the cervico-vagina then the uterine horns. Low levels of infection were detected in the tissues of the live infection control group, which confirms the strong protection against re-infection indicated by the vaginal shedding data. IN immunization with MOMP and CT/CpG was the only vaccination strategy to significantly reduce the total peak infectious burden across the entire genital tract ($P<0.05$). Interestingly, this vaccine prevented the infection from ascending to the oviducts in 2/5 mice (Figure 2A), but failed to significantly reduce the duration of chlamydial shedding (Figure 2B) or the development of pathology (Figure 1). SL and TC immunization with MOMP and CT/CpG significantly reduced the duration of vaginal shedding (Figure 2B, 100% cleared by day 18 p.i.) ($P<0.001$), but not the total burden (Figure 2A). Interestingly, immunization with MOMP and CTA1-DD via the SL route, which elicited the greatest protection against pathology (Figure 1), did not significantly reduce the total chlamydial burden in tissues at day 6 p.i or infection duration measured in the lower genital tract (Figure 2).

MOMP-specific antibodies and cytokine production by lymphocytes isolated from the spleen and MiLN

Protective immunity against *Chlamydia* induced by vaccination with MOMP is believed to be reliant on the presence of both cell-mediated and humoral responses [15]. We therefore quantified the MOMP-specific antibody and cell-mediated responses following vaccination. There was no significant association between (I) the induction of serum and mucosal antibodies or (II) their potential to neutralize *C. muridarum in vitro* (Supp Figure 1) and protection against infection or pathology. There was also no association between the cytokines produced by splenocytes following *in vitro* re-stimulation with MOMP (Supp Figure 2) and protection against infection or pathology. MOMP-specific cytokine production by lymphocytes isolated from the MiLN (Figure 3) was significantly elevated in groups

immunized with MOMP and CT/CpG that were found to elicit protection against infection and oviduct pathology. However, immunization with the MOMP and CTA1-DD by any route failed to elicit a cytokine response in the MiLN. Immunization with MOMP and CT/CpG via the SL and TC routes induced significant levels of IFNγ (Figure 3A) and TNFα (Figure 3B) ($P<0.001$) secretion that was associated with enhanced protection against both the duration of infection in the vagina and oviduct pathology. IN immunization with MOMP plus CT/CpG only elicited a significant TNFα response ($P<0.001$) and conferred protection against the ascending infection but not pathology. Production of IL-17 (Figure 3C) was the dominant cytokine detected in all groups, yet only the MOMP and CT/CpG vaccine delivered via the SL route elicited IL-17 at significant levels ($P<0.01$) compared to unimmunized controls. No vaccine induced a significant IL-10 or IL-4 response (data not shown).

Different kinetics of T cell, neutrophil and macrophage recruitment into the upper genital tract tissues of protected and unprotected animals

The protection conferred by SL and TC immunization with MOMP and CT/CpG against oviduct pathology was associated with the production of IFNγ and TNFα and a reduction in infection duration. However, protection against an ascending infection did not prevent the development of oviduct pathology (Figure 4A). IN immunization with MOMP and CT/CpG significantly reduced the infectious burden in the oviducts when compared to the unimmunized control ($P<0.05$), but could not prevent gross upper genital tract pathology (infection-protected). Interestingly, immunization with MOMP and CTA1-DD via the SL route prevented the development of hydrosalpinx, but the bacterial burden in the oviducts was not significantly different from that of the unimmunized animals (pathology-protected). We therefore sought to determine if either of these vaccination strategies altered the kinetics or magnitude of T cell ($CD3^+$) (Figure 4B and C), neutrophil ($Gr\text{-}1^+$) (Figure 4D and E) or macrophage ($F4/80^+$) (Figure 4F and G) infiltration in the upper genital tract on days 6, 12,

18 and 24 p.i. The major composition of the cellular infiltrate in unimmunized animals early during the infection was dominated by Gr-1^+ and F4/80^+ cells. Staining of tissues taken from both vaccinated groups showed an early formation of $CD3^+$ positive aggregates when compared to the unimmunized control. The infiltration of $CD3^+$ positive cells into the upper genital tract detected at day 6 p.i. in the infection-protected group immunized intranasally with MOMP and CT/CpG was also accompanied by a significant number of Gr-1^+ cells ($P<0.05$). A significant increase in the numbers of Gr-1^+ cells was also detected in unimmunized animals at day 12 p.i. ($P<0.05$), but not in the pathology-protected group immunized with MOMP and CTA1-DD over the entire course of the infection. Infiltration of F4/80^+ cells was also significant increased in all groups over the course of the infection when compared to the uninfected control, but there were no differences between groups.

Gene expression in infected oviduct tissues from protected and unprotected animals

We also analyzed the expression of key genes mediating inflammation and immunity in the oviduct tissue at day 6 p.i. This time point was chosen for analysis as it represents the peak bacterial burden in the oviducts and corresponds to a period in which the onset of irreversible pathology begins in naïve animals [16, 17]. Figure 5 depicts the gene expression profiles during a normal course of infection and pathology development (unimmunized), pathology-protected (SL delivered MOMP plus CTA1-DD) and the infection-protected (IN delivered MOMP plus CT/CpG), all normalized against the no infection control. In the unimmunized group there was a strong induction of pro-inflammatory cytokines IL-1β (63-fold increase), IL-18 (2-fold increase), TNFα (49-fold increase) and IL-6 (19-fold increase). When this gene expression profile was compared to that of an animal protected from infection exclusively (IN delivered MOMP plus CT/CpG), the infection-protected animals displayed a significant down-regulation of pro-inflammatory cytokines IL-1β (62-fold reduction), IL-18 (6-fold reduction), TNFα (48-fold reduction) and IL-6 (13-fold reduction) and up-regulation of Th17-related

190 factors IL-17A (218-fold increase), IL-17C (118-fold increase), IL-17D (11-fold increase),
191 IL-17F (864-fold increase), IL-17RC (4-fold increase), IL-17RD (8-fold increase) and IL-
192 17RE (4-fold increase). Conversely, when a normal course of infection was compared to that
193 of an animal protected from pathology exclusively (SL delivered MOMP plus CTA1-DD),
194 the pathology-protected animals displayed a significant up-regulation of Th17-related
195 cytokines IL-17A (165-fold increase), IL-17C (121-fold increase), IL-17D (8-fold increase)
196 and pro-inflammatory cytokines TNFα (5-fold increase) and IL-6 (20-fold increase). In
197 addition, these mice displayed an increased expression of Th2 and T_{reg}-associated cytokines
198 IL-13 (25-fold increase) and IL-10 (7-fold increase), respectively, and the extracellular
199 matrix hydrolyzing MMP13 (7-fold increase). These animals also showed a down-regulation
200 of the pro-inflammatory cytokines IL-1β (61-fold reduction) and IL-18 (34-fold reduction),
201 and IL-17 receptors IL-17RD (13-fold reduction) and IL-17RE (136-fold reduction).
202 Noteworthy similarities between both vaccinated groups, was the overwhelming down-
203 regulation of pro-inflammatory cytokines and over-expression of IL-17 cytokines when
204 compared to a normal course of infection. Major differences between the vaccines included
205 an increase in IL-13, IL-10, and MMP13 and a decrease in IL-17 receptor expression in the
206 pathology-protected group compared to infection protected.

Discussion

Protection against vaginal shedding following challenge was associated with the induction of MOMP-specific lymphocytes secreting TNFα and IFNγ in the draining lymph nodes. Induction of IFNγ signaling through the IFNγR is vital for the resolution of a genital tract infection, potentially through both T cell-dependent and independent mechanisms [18]. The role that TNFα plays is less clear, but it may contribute towards infection resolution as well as exacerbating pathology [19, 20]. Synergism between IFNγ and TNFα has also been reported to enhance the rate of infection clearance [21-23]. This may occur by increasing cytokine production, antigen-presentation and recruitment of T cells into the genital tract [24]. In our study, induction of both IFNγ and TNFα were crucial for protection against vaginal shedding, as IN immunization with MOMP and CT/CpG elicited TNFα only and failed to significantly reduce the duration of the infection in the lower genital tract. Therefore, the induction of IFNγ and TNFα following immunization was associated with strong protection against vaginal shedding, potentially due to a synergistic interaction between multiple pro-inflammatory cytokines.

The positioning of antigen-specific lymphocytes in the lymph nodes draining the genital tract (MiLN) was also found to associate with protection against the duration of vaginal shedding. Roan *et al*., (2006) has shown previously that the activation of *Chlamydia*-specific T cells in the MiLN and not other non-draining lymph nodes were a major correlate of protection against a *Chlamydia* genital tract infection in mice [25]. Similarly, MOMP-specific lymphocytes localized in the lymph nodes draining the lungs and not the spleen were also recently found to confer protection against *Chlamydia* respiratory tract challenge [26]. Protection against the duration of vaginal shedding conferred by the position of the antigen-specific lymphocytes in the lymph nodes draining the site of infection may have also contributed to the reduction in oviduct pathology detected in SL. and TC. MOMP and

CT/CpG immunized groups. Proliferation of antigen-specific lymphocytes, the acquisition of effector function and migratory properties can be detected in the regional lymph node draining the site of a genital herpes simplex virus infection up to two days prior to any response in the spleen [27]. Therefore, antigen-specific lymphocytes resident in the MiLN may protect against oviduct pathology by responding rapidly to the infection and limiting the involvement of the pathological innate response during infection clearance.

The ability of a vaccine to prevent *Chlamydia* from ascending the genital tract is believed to be essential for preventing infertility [28]. Interestingly, the ascension of the infection into the upper genital tract did not associate with the severity or incidence of oviduct pathology. Reports of a similar disconnection between bacterial burden and the degree of disease are not uncommon [16, 29-31]. We therefore investigated the cause of this anomaly in more detail by analyzing differences in the oviduct tissues between the vaccinated (infection- and pathology-protected) and unvaccinated groups at a time point associated with the onset of irreversible pathology [17]. The most notable differences in gene expression between vaccinated and unvaccinated animals following infection was an up-regulation of caspase-1-activated cytokines IL-1β and IL-18 in the unimmunized group. In direct contrast to IL-18, IL-1β has been extensively implicated in the development of pathology following a genital tract chlamydial infection [32-34]. Macrophages and neutrophils are the major source of IL-1β during a chlamydial infection [34], which may indicate a greater dependence on innate immunity by unimmunized mice for the clearance of infection. This is consistent with our immunohistochemical analysis of upper genital tract tissues and the findings by others that innate cell types (macrophages and neutrophils), as opposed to adaptive (T and B cells), dominate the early cellular infiltrate in the oviducts following a primary infection of naïve mice [35]. Consistent with the literature, IL-1β expression appears to associate with the development of oviduct pathology in naïve animals during a primary infection [32-34].

Both pathology- and infection-protected vaccine groups showed a significant down-regulation of IL-1β when compared to the unvaccinated group. However, the infection-protected vaccine group developed pathology, despite the reduction in IL-1β expression. This may suggest that the mechanisms mediating the development of pathology in naïve mice (IL-1β-dependent) differs from that of vaccinated animals, which may involve an adaptive element generated following vaccination. The most notable difference in gene expression between the infection-protected and unvaccinated groups was the increased expression of Th17-family cytokines (IL-17A, C, D, F, IL-17RC, D and E), which potentially indicates an increase in IL-17-mediated signaling. The cytokine IL-17A (also known as IL-17) itself does not have a direct anti-chlamydial effect, at least *in vitro* [36], and it is not crucial for host defense against a primary *Chlamydia* genital tract infection [37]. However, IL-17 and IL-17F influence the recruitment of neutrophils and development of protective Th1 immunity [38]. Yu *et al*. (2010) also recently described a correlation between the expansion of $IFN\gamma^{+}IL\text{-}17^{+}$ $CD4^{+}$ T cells following immunization and enhanced resolution of chlamydial genital tract infection [22]. The infection-protected group, shown to have elevated expression of IL-17 cytokines and their associated receptors in the oviducts, cleared the infection in the upper genital tract significantly faster than unimmunized animals that displayed almost no expression of Th17-related factors. Our data therefore may indicate a protective role for IL-17-mediated defense against a *Chlamydia* genital tract infection, at least in relation to infectious burden.

However, $IFN\gamma^{+}IL\text{-}17^{+}$ $CD4^{+}$ T cells, deemed protective against chlamydial infection [22], are also enriched in tissue affected by Crohn's disease and experimental autoimmune encephalomyelitis, suggestive of a link between Th17 and immunopathology. Lu *et al*. (2011) also recently found that protection from a chlamydial infection following vaccination correlated with high levels of IFNγ, yet a reduction in pathology required decreased levels of

IL-17 [7]. As IL-17-mediated responses have been implicated in both immunopathology and host defense against pathogens [39], it is possible that the response induced by the infection-protective vaccine limited the ascension of the infection but also caused the oviduct pathology in this group. Supportive of this hypothesis of vaccine-induced IL-17-mediated protection and immunopathology; the pathology-protected vaccine group displayed decreased expression of IL-17 cytokines and IL-17 receptors when compared to the infection-protective vaccine group. This suggests a potential suppression of IL-17-mediated signaling in the pathology-protected vaccine group that may have abrogated the development of oviduct pathology, but in doing so also inhibited the clearance of the infection in this group.

The precise mechanism(s) behind IL-17-mediated immunopathology in regards to a chlamydial infection are unknown. The role of IL-17 signaling was recently assessed in the context of a primary *Chlamydia* genital tract infection using IL-17RA-deficient mice [38]. For the most part, this study recognized IL-17-mediated responses as redundant in the development of oviduct pathology following a primary infection. We also found no association between expressions of IL-17 cytokines/receptors and oviduct disease following a primary infection, instead finding evidence to support IL-1β-induced pathology. The pathology mediated by IL-17 signaling reported in our study could have been driven by the adaptive response generated following vaccination, which would not have been present in naïve mice prior to a primary infection.

IL-17 cytokines can act upon epithelial cells as the mucosa highly expresses IL-17 receptors [40]. Stimulation of epithelial cells with IL-17 induces the release of other pro-inflammatory cytokines, which can synergize and amplify the response of IL-17 [41] to support the longevity of the pro-inflammatory immune response that causes tissue damage [42]. This response may have recruited and activated additional neutrophils, producing nitric oxides and MMPs that have also been shown to be damaging during a chlamydial genital tract infection [37, 43, 44].

307 Evidence for this response in our study can be supported by the significant increase in
308 numbers of neutrophils (Gr-1^{+}) detected in the upper genital tract in the infection-protected
309 group that exhibited increased IL-17 signaling. Moreover, IL-1β and TNFα can stimulate a
310 further release of IL-17 cytokines and expression of IL-17 receptors [45]. The induction of IL-
311 17-mediated responses following vaccination therefore may create a pathological positive
312 feedback loop, potentially initiated by the adaptive response and sustained by the host cell
313 epithelium, not unlike the cellular paradigm of chlamydial pathogenesis [46]. Further studies
314 will be required to definitively determine how IL-17-mediated responses may exacerbate
315 disease.
316 How each vaccine was able to differentially regulate IL-17-mediated responses is unclear,
317 however, the route of immunization [47] and adjuvants [48-51] utilized likely contributed. The
318 infection-protective vaccine contained both CT and CpG that elicit high levels of TGF-β, IL-
319 6 and IL-17 [48, 50], which can facilitate the development of Th17 immunity. Zygmunt *et al*.,
320 (2009) also described a predisposition for immunization via the IN route to selectively
321 promote Th17 immune responses in an adjuvant-independent manner. Alternatively, the
322 buccal mucosa targeted by the pathology-protective vaccine, which displayed reduced IL-17
323 responses, may elicit a Th17 suppressive phenotype. Both the human and mouse oral mucosa
324 express high levels TGF-β and FoxP3 and low levels of Th17 differentiation factors [52].
325 CTA1-DD also elicits a high level of IFNγ with relatively low levels of IL-17 [49], identical to
326 the type of response that is responsible for the protection against pathology [7]. The balance of
327 IL-17-mediated responses therefore can be attributed to the unique combination of adjuvant
328 and immunization route.
329 In conclusion, this study emphasized the importance of a vaccine inducing production of
330 IFNγ and TNFα and the localization of these cytokine-secreting cells in the genital tract
331 draining lymph nodes, which may protect against both the duration of infection and oviduct

pathology by exploiting a synergism between these cytokines in addition to a rapid anamnestic response. We have indicated that oviduct pathology may develop in vaccinated mice via a different mechanism to naïve mice, which could be dependent on the Th17 response. Moreover, preventing the ascension of the infection into the upper genital tract does not guarantee protection against disease and that tissue damage could be instigated by an inappropriate immune recall response generated by vaccination. Therefore, if sterilizing immunity is not possible and the final outcome of a *Chlamydia* vaccine is to eradicate disease, controlling the tissue damage instead of the infection may be the key to a vaccine against *Chlamydia* that prevents infertility.

Methods

Ethics statement

This study was approved by the Queensland University of Technology Animal Ethics Committee (Approval number 0800000432) and carried out in strict accordance with the Australian Governments National Health and Medical Research Council's guidelines for use of animals for scientific purposes.

MOMP recombinant and C. muridarum *purification*

Recombinant *C. muridarum* MOMP was purified from the *E. coli* (DH5α[pMMM3]) clone transformed with the pMAL-c2 ampicillin-resistant vector encoding the recombinant maltose-binding protein fusion protein (MOMP-MBP) as previously described [26]. *C. muridarum* (Weiss strain; ATCC VR-123) was cultured and purified as previously described [53].

Immunization protocols

Groups of five mice were immunized on days 0, 7, 14 and 28 via either IN, TC or SL routes as previously described [26]. Animals in the IN group immunized with recombinant MOMP (100 μg) and either CTA1-DD (Biovitrum) (20 μg) or CT (List Biological Laboratories) (5 μg)/CpG-ODN 1826[c] (5′-TCC ATG ACG TTC CTG ACG TT-3′) (Sigma-Aldrich) (10 μg) mixed in a 10 μL volume, 5 μL applied to each nare.

The skin of the animals in the TC group was pre-treated first with acetone, then with a solution containing dodecylpyridinium chloride (0.33% w/v), isopropyl myristate (0.33% w/v) and methyl pyrrolidone (0.33% w/v) and finally rehydrated with PBS. Mice then received granulocyte-macrophage colony-stimulating factor (GM-CSF) (12.5 ng) with recombinant MOMP (200 μg) and either CTA1-DD (20 μg) or CT (10μg)/CpG-ODN 1826[c] (10 μg) mixed in a volume of 50 μL. The immunization was contained for a 24 h time period using a patch system.

382 The SL group was immunized with recombinant MOMP (100 μg) mixed with either CTA1-
383 DD (20 μg) or CT (5 μg)/CpG-ODN 1826[c] (10μg) in a 7 μL volume was applied directly to
384 the ventral side of the tongue and left for 1 h with the head of the mice maintained in ante-
385 flexion.
386 Unimmunized controls were included for each route of immunization. These animals
387 received the exact same treatment as vaccinated mice but were mock immunized with PBS.
388 Animals were euthanized seven days after their final boost using Lethabarb® (200 mg/kg)
389 delivered IP for assessment of immune responses.
390 ***Sample Collection and Tissue Processing***
391 Vaginal lavage was taken by flushing the vagina with 40 μL of PBS collected once a day
392 over the four day estrous cycle then pooled for each animal. Blood was taken via cardiac
393 puncture, allowed to clot, after which the serum was collected by centrifugation. Uterine horn
394 lavage was collected by flushing each uterine horn with 100 μL of PBS.
395 The spleen and medial illiac lymph nodes (MiLN) [54] were excised and pooled with their
396 respective groups in complete Dulbecco's Modified Eagle Medium (DMEM), containing 5%
397 fetal calf serum (FCS), 2 mM L-glutamine, 100 μg/mL streptomycin sulphate and 2 μg/mL
398 gentamycin. These tissues were mechanically disrupted and passed through a 75 μm nylon
399 filter. All single cell suspensions treated with red blood cell lysis buffer (155mM NH_4Cl, 12
400 mM $NaHCO_3$, 100 μM EDTA, pH 7.35) for 10 min at RT. Cells finally resuspended after
401 washing in complete DMEM containing 50 μM β-mercaptoethanol.
402 ***Lymphocyte proliferation and cytokine analysis***
403 Lymphocytes from each tissue were seeded (5 x 10^5 cells/well) into a U-bottom 96-well plate
404 and stimulated with either recombinant MOMP (10 μg/well) or media alone for 72 h.
405 Following incubation a portion of the media supplementing the stimulated cells was removed
406 for analysis of cytokine production. Levels of IFNγ, IL-4, IL-17 were measured, in duplicate,

from pooled media supernatant samples using the BioPlex Mouse Express Pro Cytokine Assay 23-plex (Biorad) according to the manufacturer's instructions. TNFα levels were also detected from the pooled supernatant using the DuoSet ELISA development system (R&D systems).

MOMP-specific antibodies and in vitro neutralization of* C. muridarum *infectivity

Antigen-specific antibodies were measured by enzyme-linked immunosorbent assay (ELISA) as previously described [14], using recombinant MOMP coated (2 μg/well) plates. The endpoints (background plus two standard deviations [± 2 S.D]) were calculated for all samples using secondary order polynomials.

In vitro C. muridarum neutralization assay was conducted using a 1/10 dilution of the lavage and serum samples as previously described [26]. Percentage neutralization was determined using the equation % neutralization = [% cells infected (post-immunization) - % cells infected (pre-immunization)] / % cells infected (no sample – media).

Intravaginal* C. muridarum *challenge

Intravaginal challenge was conducted as previously described [16]. Briefly, each animal received 2.5 mg of medroxyprogesterone (DepoProvera) subcutaneously, seven days prior to challenge with *C. muridarum*. Anesthetized with ketamine and xylazine, mice were given 5 x 10^2 inclusion forming units (IFU) of *C. muridarum* in 20 μL of sucrose-phosphate-glutamine (SPG) to the vaginal vault two weeks following immunization. The no infection controls were progesterone-primed naïve mice that remained uninfected. The live infection control mice were intravaginally challenged with 5 x 10^2 IFU and after six weeks were re-challenged at the same time as the vaccinated animals with 5 x 10^2 IFU dose.

Monitoring clearance of infection and burden

Vaginal swabs were collected using a sterile nasopharyngeal Calgiswab (Modular) and stored in SPG. Animals were be deemed to have a productive infection at level ≥300 IFU per swab

[16]. Cervico-vagina, uterine horn and oviduct tissues were excised and placed in 300 μL of SPG. Tissues from the genital tract were homogenized (OMNI TH tissue homogenizer) at 5,000 rpm until tissues were completely dispersed. Chlamydial burden was quantified by culture as previously described [26].

Gross oviduct pathology

Pyosalpinx and hydrosalpinx are a collection of puss or clear fluid, respectively, in the oviducts of infected mice following scarring and occlusion of the oviducts. The swelling size of the oviducts has been shown in mice to be directly proportional to the severity of pathology and extent of the blockage [55, 56]. The incidence of oviduct pathology (presence or absence) is also commonly used as a marker of tubal factor infertility. The total number of mice in each group that develop pyo/hydrosalpinx can be divided further by the incidence of unilateral or bilateral, which indicates whether one or both oviducts are affected.

Immunohistochemistry

The uterine horns from each mouse were excised and preserved by fixing with 10% neutral-buffered formalin. Immunohistochemistry was performed by the HistoTechnology Facility – QIMR, Australia. Briefly, tissues were then embedded in paraffin, sectioned (5 μm) and dewaxed using xylene and a decreasing ethanol gradient. Endogenous peroxidase activity was blocked using 2% H_2O_2 in TBS. Heat-induced antigen-retrieval was performed in Biocare Medical Diva Antigen Retrieval (Cat. No. DV2004 LX) solution using the Biocare Medical Decloaking Chamber. Biocare Medical Background Sniper (Cat. No. BS966 G) was used for blocking non-specific antibody binding. Tissues were stained with anti-Gr-1 (Cat. No. ab2557), F4/80 (Cat. No. ab6640) (Abcam) and CD3 (Cat. No. A0452) in Da Vinci Green antibody diluent (Cat. No. ab79995) (Dako). The betazoid DAB, MACH1 (Cat. No. M1U539 G) was used to develop the signal. Stained tissue sections were scanned using the Aperio Scanscope XT histology slide scanner and analyzed using ImageScope Software (Aperio).

457 ***RNA extraction and PCR array***

458 Oviducts were excised on day 6 p.i and stored in RNAlater (QIAGEN). Total RNA was
459 extract from pooled oviduct homogenate using the RNeasy Fibrous Tissue Mini Kit (Cat. No.
460 74704) and treated with RNase-free DNase (QIAGEN). cDNA was synthesized from 1µg of
461 total RNA using RT^2 First Strand Synthesis Kit (QIAGEN) (Cat. No. 330411) as per the
462 manufacturer's instructions. cDNA samples were mixed with RT^2 SYBR ROX qPCR
463 mastermix, aliquoted into the RT^2 Profiler Mouse Th17 and Autoimmunity PCR Array
464 (QIAGEN) (Cat. No. PAMM–073) and amplified using the 7900HT FAST ABI system.
465 Fold-changes were calculated using the $\Delta\Delta^{Ct}$ method of relative quantification.

466 ***Statistical analysis***

467 All data was presented as the mean ± standard deviation. All statistics were performed using
468 GraphPad Prism® version 5.00 (GraphPad). Significant differences were determined
469 primarily using a one-way analysis of variance (ANOVA) with Tukey's post-test. Significant
470 differences were determined using a Kaplan-Meier survival curve and the log rank post-test
471 for the duration or vaginal shedding. Significance was set at $P < 0.05$ for all tests, i.e. $P > 0.05$
472 (ns), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***). P <10-fold (ns), >10-fold (*), >100-
473 fold (**) and >1000-fold (***) for expression profile analysis.

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479 ***Supplementary information***

480 Is available at Immunology and Cell Biology's website

481

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Figure Legends

Figure 1: Protection against upper genital tract pathology determined by the reduction in hydrosalpinx formation

Following an intravaginal challenge with *C. muridarum*, each group of animals was assessed for the development of oviduct pathology post-mortem at day 49 p.i. (A) The appearance of normal (unaffected) uterine horn (UH), oviduct (Ovi) and ovary (O) (left), compared to the gross upper genital tract pathology and oviduct swelling following an intravaginal infection with *Chlamydia* (right). (B) The severity of pathology was determined by measuring the diameter of the swollen oviducts. Results are presented as the mean ± SD. Significant differences were determined using a one-way ANOVA with Tukey's post-test. (C) The incidence of oviduct pathology was presented as percentage of animals with developing hydrosalpinx (total, bilateral and unilateral). Significant differences were determined using a Fisher's exact test. Significance was set at P >0.05 (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

Figure 2: Protection against infection determined by the reduction in infectious burden in the tissues of the genital tract and the duration of vaginal shedding

(A) Total infectious load detected in the vaginal swab, cervico/vagina, uterine horns and oviducts samples was determined at day 6 p.i. The chlamydial IFU was calculated per swab or per mg of host tissue. Results are presented as the mean ± SD. Significant levels of bacterial burden were determined using a one-way ANOVA with Tukey's post-test. (B) Percentage of animals with a detectable vaginal *C. muridarum* infection. Following an intravaginal challenge with *C. muridarum*, vaginal swabs were collected over the entire duration of the infection. Animals were deemed to have productive infection at ≥300IFU/swab. Results are presented as percentage of animals with a productive infection. The heat map represents 100% of animals infected in black, grey as animals begin to clear the infection and white when 100% of animals had no detectable infection. Significant differences were determined using a Kaplan-Meier survival curve and the

log rank post-test. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

Figure 3: Cytokine production by lymphocytes isolated from the MiLN following stimulation with MOMP

Lymphocytes isolated from the MiLN draining the genital tract were stimulated with MOMP or media for 72hr. The amount of (A) IFNγ, (B) TNFα, and (C) IL-17 (pg/mL) secreted by the lymphocytes were quantified using Bioplex and ELISA. Results are presented as the mean ± SD. Significant differences were determined using a one-way ANOVA with Tukey's post-test. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

Figure 4: T cell, neutrophil and macrophage recruitment into the upper genital tract tissues of protected and unprotected animals

(A) The incidences of oviduct swelling (either pyosalpinx or hydrosalpinx) were determined for SL. MOMP + CTA1-DD (pathology-protected), IN. MOMP + CT/CpG (infection-protected) and unimmunized groups on days 6, 12, 18 and 24 p.i (bar graph – right y-axis). Significant differences were determined using a Fisher's exact test. The chlamydial load was also determined from homogenates of the oviducts at each time point using culture and results were expressed as chlamydial IFU per mg of tissue (line graph – left y-axis). Significant differences were determined using a one-way ANOVA with Tukey's post-test. The upper genital tract, consisting of the uterine horn and the oviduct, was collected from SL. MOMP + CTA1-DD (pathology-protected), IN. MOMP + CT/CpG (infection-protected) and unimmunized groups at day 6, 12, 18 and 24 p.i. Serial tissue sections of the lumen were stained for (B) $CD3^+$, (D) Gr-1^+ and (F) $F4/80^+$ cells (representative images are shown for each). ImageScope software was used to quantify the number of positive pixels verse total pixels from high resolution images from (C) $CD3^+$, (E) Gr-1^+ and (G) $F4/80^+$ stained slides. Significant differences were determined using a

716 one-way ANOVA with Tukey's post-test. All results are presented as the mean ± SD.
717 Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**)
718 and <0.001 (***).

719 **Figure 5: Gene expression of key mediators of inflammation and immunity in the**
720 **oviducts of protected and unprotected animals at day 6 p.i**

721 The RNA was extracted from pooled oviducts of the SL. MOMP + CTA1-DD (pathology-
722 protected), IN. MOMP + CT/CpG (infection-protected), unimmunized (unprotected) and no
723 infection control groups at day 6 p.i. The difference in expression of T cell surface receptors,
724 suppressor of cytokine signaling (SOCS), pro-inflammatory cytokines, Th1/Th2/Th17/T_{reg}
725 differentiation factors and MMPs between pathology-protected animals, infection-protected
726 animals and unimmunized were determined by RT-PCR. Expressions for all groups were
727 normalized against the no infection control group. Results are presented as the mean fold-change
728 for five mice. Significance was set at >10-fold change. Between 10 – 100-fold-change (*), 100 –
729 500-fold-change (**) and >500-fold-change (***).

730 **Supplementary Figure 1: Antigen-specific systemic and mucosal antibodies**

731 (A) MOMP-specific serum IgG, IgA IgG1 and IgG2a and following vaccination was quantified
732 by direct ELISA. Endpoint titers were calculated for all samples using background absorbance of
733 PBST plus two SD. The ratio of IgG2a:IgG1, used to determine Th1:Th2 polarization, is
734 indicated above the titer bars in each group. (B) Percentage of infection neutralized *in vitro* was
735 determined by incubation of *Chlamydia* with a 1/10 dilution of whole serum. MOMP-specific
736 IgG and IgA antibodies from the (C) vaginal lavage and the (D) uterine horn lavage were also
737 quantified as above. Results are presented as the mean ± SD. Significant differences were
738 determined using a one-way ANOVA with Tukey's post-test. Significance was set at $P < 0.05$
739 for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).

Supplementary Figure 2: Cytokine production by lymphocytes isolated from the spleen following stimulation with MOMP

Splenocytes were stimulated with MOMP or media for 72hr. The amount of (A) IFNγ, (B) TNFα, and (C) IL-17 (pg/mL) secreted by the splenocytes were quantified using Bioplex and ELISA. Results are presented as the mean ± SD. Significant differences were determined using a one-way ANOVA with Tukey's post-test. Significance was set at $P < 0.05$ for all tests. $P > 0.05$ (not shown), 0.01-0.05 (*), 0.001-0.01 (**) and <0.001 (***).