Title: Induction of partial immunity in both males and females is sufficient to protect females against sexual transmission of *Chlamydia*

Running title: Male and female immunity synergize for protection

Authors and affiliations: Connor P. O'Meara[1,2], Charles W. Armitage[1], Avinash Kollipara[1], Dean W. Andrew[1,3], Logan Trim[1], Mark B. Plenderleith[4] and Kenneth W. Beagley[1]

[1]*Institute of Health and Biomedical Innovation (IHBI), Queensland University of Technology (QUT), Brisbane, Queensland, Australia*

[2] *Department of Developmental Immunology, Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Baden-Württemburg, Germany*

[3] *Centre for Biomedical Research, Burnet Institute, Melbourne, Victoria, Australia*

[4]*Neuroscience Laboratory – School of Biomedical Science, Queensland University of Technology (QUT), Brisbane, Queensland, Australia*

Conflict of Interest Statement

No conflict

Acknowledgements

This work was supported by the Queensland Government Smart State National and International Research Alliances Program, Australia-Canada-India Chlamydia Research Alliance, National Health and Medical Research Council (553055 and 1049571) and the Michael Smith Foundation Post-Doctoral Fellowship, and we thank Peter Timms for his contribution towards acquiring this funding. We thank Donna West and Jo-Anne Finnimore from QUT for their assistance with animal monitoring/housing and cytospin/Geimsa staining, respectively. We thank Genentech Inc. for the purified αCD20 antibody. We would also like to thank Clay Winterford and Nigel Waterhouse at the HistoTechnology Facility – Queensland Institute of Medical Research (QIMR) Berghofer Medical Research Institute for performing the immunohistochemistry and slide scanning.

Keywords: Animal model, *Chlamydia*, mouse model, resident memory T cell, sterilizing immunity, vaccine

Address for correspondence

Dr. Kenneth W. Beagley

Institute of Health and Biomedical Innovation

Queensland University of Technology

60 Musk Ave, Kelvin Grove, QLD 4059

AUSTRALIA

Tel: +617-3138 6195

FAX: +617-3138 6030

Email: k2.beagley@qut.edu.au

Abstract

Sexually transmitted *Chlamydia trachomatis* causes infertility, and because almost 90% of infections are asymptomatic, a vaccine is required for its eradication. Mathematical modeling studies have indicated that a vaccine eliciting partial protection (non-sterilizing) may prevent *Chlamydia* infection transmission, if administered to both sexes prior to an infection. However, reducing chlamydial inoculum transmitted by males and increasing infection resistance in females through vaccination to elicit sterilizing immunity has yet to be investigated experimentally. Here we show that a partially protective vaccine (chlamydial major outer membrane protein – MOMP and ISCOMATRIX – IMX) provided sterilizing immunity against sexual transmission between immunized mice. Immunizing male or female mice prior to an infection reduced chlamydial burden and disease development, but did not prevent infection. However, infection and inflammatory disease responsible for infertility were absent in 100% of immunized female mice challenged intravaginally with ejaculate collected from infected immunized males. In contrast to the sterilizing immunity generated following recovery from a previous chlamydial infection, protective immunity conferred by MOMP/IMX occurred independent of resident-memory T cells. Our results demonstrate that vaccination of males or females can further protect the opposing sex, whereas vaccination of both sexes can synergize to elicit sterilizing immunity against *Chlamydia* sexual transmission.

Introduction

In Australia, *C. trachomatis* accounts for 81% of all notifiable sexually transmitted infections and cases have increased from 14,045 in 1999 to 74,305 in 2010 (1). Women are 2 to 3 times more likely than men to contract an infection (2) and also have a higher incidence of asymptomatic infection, 70 – 90% compared to 40 – 60% in men (3). As the financial burden of *Chlamydia* infection is determined by the ease and cost of diagnosis, treatment and management of associated diseases (e.g. infertility), women account for 80% of the total economic cost of *Chlamydia* infections (4). Consequently, developing a vaccine that protects females from infection and disease has been the primary focus, despite studies showing the benefits of targeting both sexes (5).

We are reliant on animals to model infection transmission, and chlamydial research is most commonly conducted using the murine model, in which female mice require progesterone pretreatment to enhance their susceptibility to infection (6). Unfortunately, female mice become less receptive to mating during progesterone-induced diestrus (7), which prevents the study of sexual transmission between infected males and infection-susceptible female mice. Researchers instead utilize direct intravaginal inoculation of progesterone-primed female mice with *Chlamydia* to model sexual transmission. However, the infective dose can be highly variable between research groups, which can influence the kinetics of infection, immunity and disease development (8, 9). The inoculums of *Chlamydia* often used to infect mice are also considerably higher than the amount identified in guinea pigs and humans to be passed naturally through sexual transmission (10-12). Direct intravaginal inoculation of female mice with *Chlamydia* in sucrose buffer also cannot replicate the effects male ejaculate has on female genital tract immunity (13). Therefore, there is a need to improve the current model of *Chlamydia* sexual transmission in mice to test intervention strategies like vaccines.

The chlamydial major outer membrane protein (MOMP) is the most commonly used antigen in chlamydial vaccine research (14). However, the superior quality of MOMP-specific immunity that develops following a natural infection with *Chlamydia*, as opposed to simple immunization with MOMP, suggests that current adjuvant technology is failing to fully exploit the protective capabilities of MOMP. Similar in structure to ISCOMs (15), ISCOMATRIX® (IMX) is simply mixed with the antigen but does not need to encapsulate it to have an adjuvant effect, as the addition of phosphatidyl (choline or ethanol amine) into IMX during manufacturing provides exposed functional groups that allows for antigen conjugation through non-covalent interactions. IMX is safe and well-tolerated in humans and shown to elicit long-lived T and B cell responses in clinical trials (15). As protection against a chlamydial genital tract infection is dependent on effective antigen presentation by dendritic cells and the induction of both T and B cells (16), the combination of MOMP and IMX together with intranasal (IN) administration to elicit a mucosal response could formulate an effective vaccine against a genital tract infection.

By challenging infection-susceptible (progesterone-primed) female mice with ejaculates collected from infected males we could simulate *Chlamydia* sexual transmission in mice. Immunization with MOMP/IMX elicited partial protection against infection in both males

and females; however, infection and disease were completely absent from immunized female mice challenged with infectious prostatic fluid collected from immunized males, indicating a synergism between the partial immunity in both sexes.

Results

MOMP-specific T and B cell responses generated following immunization

A MOMP-specific cell-mediated and humoral immune response contributes significantly towards protection against a *Chlamydia* genital tract infection (17). We therefore isolated splenocytes from immunized mice and quantified the expansion and cytokine production of their T helper cells (Th) (CD3+CD4+) and cytotoxic T cells (Tc) (CD3+CD8+) (Supplementary Figure 1) cells following *in vitro* re-stimulation with MOMP. Th, but not Tc cells isolated from MOMP/IMX immunized animals showed a significantly higher level of proliferation following re-stimulation with MOMP ($p<0.001$) when compared to the unimmunized control (Fig. 1A). MOMP-specific Th cells isolated from MOMP/IMX immunized mice also showed significant staining for IFNγ ($p<0.001$), TNFα ($p<0.001$), IL-17 ($p<0.001$) when compared to unimmunized controls (Fig. 1B). Similarly, Tc cells displayed significant staining for IFNγ ($p<0.05$) and IL-17 ($p<0.001$) when compared to unimmunized controls. Further analysis of the MOMP-specific Th cells isolated from the MOMP/IMX immunized animals indicated that approximately 60% were multifunctional (IFNγ+TNFα+IL-17+, TNFα+IL-17+ IFNγ+IL-17+, $p<0.001$) compared to <10% in the unimmunized control groups (Fig: 1C). Tc cells in MOMP/IMX immunized mice were primarily of the single-positive phenotype (IFNγ+, IL-17+ or TNFα+) and not significantly different from the unimmunized control. Immune responses generated following immunization were identical in male and female mice (data not shown).

Immunization with MOMP/IMX generated MOMP-specific sera and vaginal IgG and IgA (Fig 1D) and these were significantly greater than those detected following an intravaginal infection (LIC, $p<0.01$). Sera from MOMP/IMX immunized mice significantly neutralized *Chlamydia* infectivity *in vitro* when compared to naïve sera ($p<0.05$) (Fig. 1E). Stimulation of RAW264.7 with *Chlamydia* opsonized with sera from MOMP/IMX immunized also promoted expression of CXCL1 (7-fold), CXCL2 (1.5-fold) and IL-1β (2-fold) mRNA (Fig 1F) after 24 hrs of stimulation.

Determining the infectious dose of Chlamydia *transmitted by male mice*

The bacterial burden in the prostatic fluid, prostate and testes remained constant from week 1 to week 10 post-infection (p.i) (Fig. 2A and B). The bacterial burden in the penis peaked at week 10 p.i, although this had little effect on the total amount of viable *Chlamydia* collected in the prostatic fluid over the course of the infection. Males produced 4.7 ± 1.7 μL (BALB/c) and 4.4 ± 2.4 μL (C57BL/6) of prostatic fluid (data not shown). The chlamydial load in the prostatic fluids contained 49 ± 20 IFU (BALB/c) and 64 ± 35 IFU (C57BL/6) of *Chlamydia*. Infected males were mated with receptive females (non-progesterone primed) at weeks 1 and 2 p.i and the transmissible chlamydial load in their ejaculate was determined indirectly from the females (Fig. 2C and D). Samples collected from female mice that were mated with males at week 1 p.i contained the most *Chlamydia* IFU. Vaginal lavages collected from mated BALB/c females contained 251 ± 27 IFU (♂BALB/c) and 258 ± 34 IFU (♂C57BL/6), whilst

the vaginal plugs contained 225 ± 153 IFU (♂BALB/c) and 165 ± 50 IFU (♂C57BL/6) of *Chlamydia*. For both direct and indirect sample collection methods, the transmissible dose was determined to be 18 – 503 IFU/ejaculate (Supplementary Figure 2).

***Protection against infection and disease in female mice following challenge with sexually transmissible doses of* Chlamydia**

Vaginal shedding was significantly reduced in MOMP/IMX immunized animals challenged with $5x10^1$ IFU on day 3 (15-fold) ($p<0.001$) and 6 p.i (5-fold) ($p<0.001$) when compared to the unimmunized control group (Fig. 3A). All unimmunized control animals challenged with $5x10^1$ IFU of *C. muridarum* developed a cultivatable vaginal infection, whereas, 20% of immunized animals remained culture negative from the point of challenge up until days 12 – 15 days post-challenge. This suppressive effect on infection in the vagina was abrogated when immunized animals were challenged with doses of $1x10^2$ IFU or greater. However, the duration of infection and chlamydial shedding was still significantly reduced in the vagina of MOMP/IMX immunized animals when compared to the unimmunized control groups on day 6 p.i (3-fold) when mice were challenged with $1x10^2$ IFU ($p<0.001$) (Fig. 3B), as well as on day 3 (4-fold) ($p<0.001$) when mice were challenged with $5x10^2$ IFU (Fig. 3C). Immunized mice began clearing the infection in the lower genital tract from day 9 – 15 p.i, compared to day 21 – 35 p.i for the unimmunized group. Immunization elicited a significant level of protection against the severity of oviduct pathology following challenge with $5x10^1$ ($p<0.001$) (Fig. 3A), $1x10^2$ ($p<0.001$) (Fig. 3B) and $5x10^2$ ($p<0.01$) (Fig. 3C), when compared to unimmunized controls. Mice recovered from a previous intravaginal chlamydial infection elicit sterilizing immunity against re-challenge. A prior infection (live infection control – LIC) elicited sterilizing immunity against all challenge doses, but no protection against the development of oviduct pathology.

***Immune response in the genital tract of female mice following challenge with sexually transmissible doses of* Chlamydia**

By isolating cells from the entire genital tract (sans ovaries) (Fig. 4), we could identify differences in immune cell recruitment between immunized (MOMP/IMX), unimmunized (PBS) and immune mice (LIC) and link each cell infiltrate profile with its respective infection/disease protection profile. Leukocytes (CD45+) (Fig. 4A), neutrophils (CD45+Gr-1+) (Fig. 4B), macrophages (CD45+F4/80+) (Fig. 4C), B cells (CD45+CD19+) (Fig. 4D) Th cells (CD45+CD3+CD4+) (Fig. 4E) and Tc cells (CD45+CD3+CD8) (Fig. 4F) were analyzed (Supplementary Figure 3). Recruitment of B, Th and Tc cells into the genital tracts occurred on day 8 following challenge in both immunized and unimmunized animals. Although numbers of B, Th and Tc cells recruited to the genital tract were consistently greater in immunized versus unimmunized mice, levels were not significantly different between groups. Tc cells were however present in significant numbers in the genital tract of the LIC group prior to re-challenge (day 0) ($p<0.001$) and on days 2 and 4 p.i ($p<0.05$) when compared to the unimmunized controls. Th cell numbers were also significantly increased in the female genital tracts of mice in the LIC when compared to unimmunized mice on days 2 and 4 ($p<0.05$) following challenge, indicating a rapid recruitment of Th cells in the LIC group. Neutrophil and macrophage numbers were not significantly different between immunized and

unimmunized mice. Uterine horn tissue sections were also stained for neutrophils (Gr-1+) (Fig. 4G), macrophages (F4/80+) (Fig. 4H), B cells (B220+) (Fig. 4I) and T cells (CD3+) (Fig. 4J), which matched those determined by flow cytometry. Interestingly, 8 – 15% of Tc cells isolated from the genital tract of mice from the LIC on day 2 p.i were also CD103+, indicating they were tissue resident memory T cells (T_{RM}) (18) (Fig. 5). However, T_{RM} (CD3+CD8+CD103+) diminished to background levels within one week of the secondary challenge.

Gene expression of immune cells sorted from the genital tract of female mice following challenge with a sexually transmissible dose of Chlamydia

As no significant differences in the anamnestic response were observed in the genital tract between immunized and unimmunized female mice, we assessed gene expression in whole oviduct tissues (Fig. 6A) and leukocytes recruited into the genital tract, sorted (Supplementary Figure 3) into macrophages (Fig. 6B), neutrophils (Fig. 6C), Tc cells (Fig. 6D) and Th cells (Fig. 6E) to identify functional differences. Th and Tc cells isolated on day 8 p.i from MOMP/IMX immunized mice expressed significantly more IFNγ (8- and 6-fold, respectively), TNFα (2- and 6-fold, respectively) and IL-17A (10- and 12-fold, respectively) than unimmunized mice. Similarly, Th and Tc cells isolated on day 2 p.i from LIC mice expressed significantly more IFNγ (6- and 2-fold, respectively), TNFα (2- and 3-fold, respectively) and IL-17A (3- and 13-fold, respectively) than unimmunized mice on day 8 p.i. Neutrophils and macrophages isolated on day 8 p.i from MOMP/IMX immunized mice expressed significantly more CXCL1 (2-fold) and IL-1β (2- and 4-fold, respectively) than unimmunized mice. Similar increases in gene expression were also detected in the whole oviduct gene expression. This pattern of cytokine and chemokine expression in the oviduct of MOMP/IMX immunized mice on day 2 p.i was similar to the gene expression RAW264.7 cell line stimulated with opsonized *C. muridarum* (Fig 1F).

In vivo *depletion and passive immunization*

To confirm the roles of T and B cells in protection in female mice, we depleted Th (CD4+), Tc (CD8β+) and B cells (CD20+) following immunization but prior to challenge. Th, Tc and B cells (Supplementary Figure 4) were successfully depleted from the spleen and the genital tract following administration of αCD4, αCD8 and αCD20, respectively. MOMP-specific Ig was not reduced in the serum or vaginal lavages of MOMP/IMX immunized mice following B cell depletion (Supplementary Figure 4). Naïve mice were also passively immunized with sera collected from MOMP/IMX immunized mice. Serum antibody levels were found to be comparable between passively immunized and MOMP/IMX immunized mice (Supplementary Figure 4). Depletion of CD4+ and CD8+ but not CD20+ cells from MOMP/IMX immunized mice prior to challenge, inhibited protection against infection and oviduct pathology (Fig. 7A). Depletion of CD4+ but not CD8+ or CD20+ cells from unimmunized mice prior to challenge inhibited the natural resolution of the infection, but did not exacerbate oviduct pathology (Fig. 7B). Depletion of CD8+ and CD4+ but not CD20+ cells prior to re-challenge mitigated sterilizing immunity in LIC mice, but did not exacerbate oviduct pathology (Fig. 7C). Passive immunization of naïve mice with serum from

MOMP/IMX reduced vaginal shedding between days 3 and 6 p.i when compared to infusion of sera collected from unimmunized mice, but did not affect oviduct pathology (Fig. 7D).

Protection against **Chlamydia** *infection transmission between male and female mice*

Immunization conferred partial protection against infection in female mice, although it is unclear if vaccinating males prior to their infection could prevent or reduce the risk of infection transmission to immunized female mice. Unimmunized female mice challenged with the prostatic fluid collected from unimmunized males (week 1 p.i) developed a comparable vaginal infection and disease to females challenged with known doses of $5x10^1$, $1x10^2$ and $5x10^2$ IFU of *C. muridarum* (Fig. 8A). Immunization of male mice prior to challenge significantly reduced the amount of *Chlamydia* detectable in the penis and testes, but not in prostatic fluid (Supplementary Figure 5). However, unimmunized female mice challenged with the infectious prostatic fluids from immunized males exhibited no reduction in infection or disease when compared to infection with unimmunized male ejaculates (Fig. 8B). Half of immunized female mice showed sterilizing immunity against challenge with the infectious prostatic fluid from unimmunized males ($p<0.001$), but these mice showed no protection from disease when compared to challenge of unimmunized females (Fig. 8C). Interestingly, no immunized female mice challenged with the infectious ejaculate of immunized males acquired an infection or developed any oviduct pathology ($p<0.001$) (Fig. 8D), indicating complete protection against *Chlamydia* sexual transmission between immunized males and females.

Discussion

The ability of an animal model to replicate sexual transmission of infection is crucial for the development of a human vaccine against *Chlamydia*. To date, only large and/or expensive animal models (non-human primates, pigs and guinea pigs) have been shown to transmit a chlamydial infection sexually (16). In this study, we determined the transmissible infectious dose in the ejaculate of male mice (18 to 503 IFU) in order to develop a mouse model of sexual transmission. This dose range determined for mice is comparable with Guinea pigs that transmit approximately 100 IFU of *C. caviae* (11, 12). Semen from *C. trachomatis*-infected human males has also been shown to contain between 675 – $1.6x10^4$ copies/mL of chlamydial DNA (10). With an average ejaculate volume of 4.66 mL (10), a transmissible infectious dose for humans would range between 3146 – 7.5 $x10^4$ copies/ejaculate, which closely matches the amount contained in mouse prostatic fluid (2080 – $1.16x10^5$ copies/prostatic fluid). Therefore, we suggest that an inoculum of *C. muridarum* between 50 and 500 IFU should be used for all future vaccine studies as this dose is not only capable of establishing an infection in all test animals, but it also reflects a physiologically relevant inoculum that could be passed naturally during sexual transmission.

Surprisingly, 20% of immunized female mice initially resisted the establishment of a genital tract infection for 12 – 15 days post-challenge with the sexually transmissible dose of 50 IFU. Despite the expansion of MOMP-specific and multifunctional Th and Tc cells following immunization, these could not be detected in the genital tract until day 8 p.i. Conversely, significant levels of MOMP-specific antibodies were generated following immunization, which were also shown to neutralize *Chlamydia* infectivity *in vitro* and reduce vaginal

shedding on days 3 and 6 p.i following infusion into naïve animals. In support of finding by others (19), this indicates that induction of humoral immunity through immunization is crucial for neutralizing the initial challenge inoculum, an important effect that was masked when using higher, non-physiological challenge doses of *C. muridarum* (>5x10^3 IFU) (data not shown).

Expression of chemokines (CXCL1, CXCL2 and CXCL5) and cytokines (IFNγ, TNFα and IL-1β) were up-regulated in the oviducts of immunized compared to unimmunized mice. Macrophages and neutrophils isolated from the genital tracts of immunized mice also showed a similar up-regulated pattern of cytokine and chemokine expression as the oviducts, indicating an increased level of activation, antimicrobial and chemotactic activity (16). This occurred during the early stages of the infection (day 2 p.i) where the presence of MOMP-specific antibodies in the genital tract of immunized animals was the only observed difference from unimmunized mice. Opsonization and complement are known to promote chemokines and cytokine production by APCs (20) and we too found that *Chlamydia* incubated with sera containing MOMP-specific antibodies enhanced expression of cytokines and chemokines by monocyte/macrophages *in vitro*. This indicated that in addition to their neutralizing effects, antibodies generated following immunization might also contribute towards protection by enhancing innate and adaptive responses through the induction of cytokines and chemokines.

The recruitment of T cells into the genital tract on day 8 p.i coincided with the resolution of the infection in immunized animals. However, unimmunized mice failed to resolve an infection from day 8 p.i despite mounting a T cell response comparable in both speed and magnitude to immunized animals. This indicated that functional differences existed between T cells recruited in immunized and unimmunized animals. Immunization induced expansion of MOMP-specific splenic Th and Tc cells, which produced IFNγ, TNFα and IL-17A upon *in vitro* re-stimulation. Th and Tc cell isolated from the genital tracts of immunized mice on day 8 p.i were also found to express more IFNγ, TNFα and IL-17A than T cells isolated from unimmunized animals. These cytokines have been consistently shown to contribute to protection against infection and disease (21). Protection against vaginal shedding was alleviated following depletion of CD4+ and CD8+ cells, indicating an important role for Th and Tc cells in protection elicited by MOMP/IMX.

Animals recovered from a previous infection with *Chlamydia* (LIC group) demonstrated sterilizing immunity against re-challenge; therefore, it is important to compare the mechanisms of protection between MOMP/IMX immunized and LIC mice as this can highlight potential inadequacies in vaccine design. T cells were not detectable in the genital tract of MOMP/IMX immunized until day 8 p.i, whereas Tc cells were present in the genital tract of LIC mice prior to re-challenge and Th cells were rapidly recruited by day 2 p.i. Some Tc cells present in the genital tract of LIC mice prior to challenge were CD103+ and localized in the epidermis, which is consistent with the phenotype of T_{RM} in female genital tract of mice during a HSV-2 infection (22). However, T_{RM} we not detected in the genital tract of MOMP/IMX immunized mice even following challenge (day 2 – 8 p.i.). Tc cells isolated from the genital tracts of mice in the LIC group immediately prior to re-challenge (day 2 p.i) expressed increased levels of cytokines, which were associated with the rapid

recruitment of Th cells. This is also consistent with existing literature regarding the functionality of T_{RM}, that they provide a means of "sense and alarm" (23) by secreting cytokines and chemokines, which recruit effector Th cells necessary to eradicate the infection. Th and Tc cells recruited in the genital tract of MOMP/IMX immunized mice exhibited a similar pattern of increased cytokine expression as T cells from LIC, but were recruited later at day 8 p.i. Reduction of Tc cells numbers, including the T_{RM}, in the genital tract of LIC mice prior to re-challenge abrogated sterilizing immunity, which indicated that Tc cells are capable of preventing infection from sexually transmissible doses of *Chlamydia*. Th cells were also shown to contribute to sterilizing immunity in LIC mice, however; it is unclear if Th cells had a direct effect or if their depletion during the resolution of the primary infection inhibited the development of T_{RM} (24). Further phenotypic characterization, functionality and longevity studies will also be required to definitively characterize T_{RM}, although this to our knowledge is the first indication that a small number of T_{RM} may be generated following a chlamydial infection. Comparison between immunized and LIC mice indicate that strategies to accelerate T cell recruitment into the genital tract, like the "prime-pull" approach (25), must be implemented if future vaccines are to achieve immunity equivalent to that which develops following a natural infection.

Mathematical modeling has been invaluable in determining the attributes a vaccine must possess in order to impact on infection transmission (5, 26). Assertions that non-sterilizing vaccines, if targeted towards both sexes, could interrupt infection transmission had not yet been investigated experimentally (5). Challenging female mice with prostatic fluids collected from infected males to model sexual transmission (Fig. 9A), we determined that the immunization status (immunized or unimmunized) of both sexes was critical for the induction of sterilizing immunity against infection and disease. Immunizing females was more effective against preventing infection transmission than immunizing males (Fig. 9B and C), as reported elsewhere (5). This could be attributed to the inherent difficulties in resolving an infection in the immune-privileged male genital tract and the vaccine eliciting a potent cell-mediated response as opposed to the more male protective humoral response (27). However, male vaccination status protected against the development of oviduct pathology in immunized females (Fig. 9D). As this reduction in severity and incidence of disease was not detected in unimmunized females, it is likely to be independent of bacterial burden. Instead, additional adaptive immune components like antibodies, chemokines or cytokines present in the ejaculate of immunized males may have provided cross talk with immunity in immunized females (13, 28). This indicates that anti-chlamydial immunity against infection will be dependent on a vaccine for females, whereas a vaccine targeting males may be required for protection against diseases associated with infertility.

In this study, analysis of the local immune response in the genital tract following a *Chlamydia* infection provides the first evidence that T_{RM} confer/are essential for sterilizing immunity against challenge and should be the target of future vaccines. Induction of T_{RM} in females may be required to elicit complete protection against an infection transmitted from an unimmunized male. A partially protective vaccine targeting females would have a greater impact on *Chlamydia* infection and disease than a male vaccine; however, sterilizing immunity, i.e. prevention of sexual transmission, would require vaccination of both sexes. It

has been consistently shown in the most at risk adolescent age group that sexual behavior and perception of risk to other sexually transmitted infections is unaltered by vaccination (29); therefore, the introduction of a non-sterilizing *Chlamydia* vaccine together with existing surveillance and antibiotic treatment programs could facilitate a decline in infection rates.

Materials and Methods

Ethics statement

Animal care and use protocols in this study adhered to the National Health and Medical Research Council guidelines to promote the wellbeing of animals used for scientific purposes. This study was approved by the Queensland University of Technology Animal Ethics Committee (QUT UAEC No. 1100000588 and 1400000010) and carried out in strict accordance with any recommendations. All animals were euthanized humanely by intraperitoneal injection with sodium pentobarbital (200 mg/kg).

Animals and Chlamydia

Mice (C57BL/6 and BALB/c) were sourced from the Animal Resource Centre (ARC, Canningvale, Australia) at 6 weeks of age. Animals were given food and water *ad libitum*. *C. muridarum* (Weiss strain), was a generous gift from Catherine O'Connell and cultured from McCoy cells and purified as previously described (30).

Immunization

Recombinant *C. muridarum* MOMP-MBP was purified and IN immunizations were performed as previously described (31). The vaccine contained recombinant MOMP (50 μg) and ISCOMATRIX® (10 μg) (Zoetis, Florham Park, USA) mixed in a 10 μL volume, 5 μL applied to each nare. Animals were immunized once on four separate occasions (days 0, 7, 14 and 28).

Antibodies and flow cytometry

Cells were washed with 2%v/v fetal calf serum (FCS)/phosphate-buffered saline (PBS) before blocking with αCD16/CD32 – FcγR (Clone: 24G2) for 15 min at 4°C. Cells were labeled with Zombie Green Viability Dye (Cat No. 423112, Biolegend, San Diego, USA) prior to staining with αCD3 (Clone: 145-2C11, Cat No 100330 – Biolegend, San Diego, USA), αCD4 (Clone: RM4.5, Cat No. 562314 – BD Bioscience, San Jose, USA), αCD8α (Clone: 53.6-7, Cat No. 25-0081 – eBioscience, San Diego, USA), αCD19 (Clone: 1D3, Cat No. 551001 – BD Bioscience), αCD45 (Clone: 30-F11, Cat No. 562129 – BD Bioscience), αCD103 (Clone: 2E7, Cat No. 121408 – Biolegend), αGr-1 – Ly6C/Ly6G (Clone: RB6-8C5, Cat No. 108412 – Biolegend) and αF4/80 (Clone: BM8, Cat No. 123110 – Biolegend) in a volume of 50 μL for 15 min at 4°C. Cells were washed with 2% FCS/PBS before fixing using 4%w/v paraformaldehyde for 10 min at 4°C. Fixed cells were permeabilized for intracellular cytokine staining using Perm/Wash (Cat No. 554723 – BD Bioscience) according to the manufacturer's instructions. Permeabilized cells were stained with αIFNγ (Clone: XMG1.2, Cat No. 554413 – BD Bioscience), αTNFα (Clone: MP6-XT22, Cat No. 554418 – BD Bioscience) and αIL-17 (Clone: TC11-18H10, Cat No. 559502 – BD Bioscience) diluted in Perm/Wash buffer for 40 min at 4°C. Stained cells were analyzed on the FACSAria III (BD Bioscience) using Flowjo version X software (Tree Star Inc, Ashland, USA).

In vivo *depletion and passive immunization*

Depletion of CD4+ (Clone: GK1.5, IgG2b, 1 x 200 µg), CD8β+ (Clone: 53-5.8 (32), IgG1, 1 x 250 µg IP, 2 x 50 µg intravaginally) (BioXcell, West Lebanon, USA) and CD20+ cells (Clone: 5D2, IgG2a, 1 x 250 µg) (Genentech Inc, San Francisco, USA) was performed following IP injection of each monoclonal antibody on day 13 prior to intravaginal challenge. Identical doses of αTNP (Clone: 2A3, IgG2a), αHRP (Clone: HRPN, IgG1) or αKLH (Clone: LTF-2, IgG2b) (BioXcell) were used as isotype controls. Passive immunization of naïve mice was performed one day prior to intravaginal challenge by IP infusion of 500 µL of serum collected from MOMP/IMX or PBS immunized mice.

Sample collection and tissue processing

Vaginal lavages and serum were collected as previously described (21). Spleens processed as described previously (21) and cultured in complete Dulbecco's minimal essential medium (DMEM) (5%v/v fetal calf serum (FCS), 4 mM L-glutamine, 50 µg/mL gentamicin, 100 µg/mL streptomycin sulfate) (Invitrogen, Carlsbad, USA) containing 50 µM β-mercaptoethanol. Genital tract tissues were digested in complete DMEM containing 500 U/mL of collagenase I and 120 µg/mL DNase I (Invitrogen) for 1 hr shaking at 37°C.

MOMP-specific T cell proliferation and cytokine production

Cells were stained with carboxyfluorescein succinimidyl ester (CFSE) (Sigma-Aldrich, St. Louis, USA) (5 µM) for 5 min at room temperature. Labeled splenocytes were stimulated with media containing recombinant MOMP (10 µg/well) for 96 hrs at 37°C with 5% CO_2. Cells were incubated for an additional 12 hrs in complete DMEM containing brefeldin A (Sigma-Aldrich) (10 µg/mL) before intracellular cytokine staining.

MOMP-specific antibody quantification, in vitro *neutralization of* Chlamydia *infectivity and opsonization*

Antigen-specific antibodies were measured by enzyme-linked immunosorbent assay (ELISA) and *in vitro C. muridarum* neutralization assay were performed as previously described (21).

Monocytes/macrophage cell line RAW264.7 (ATCC TIB–71) were grown in RPMI 1640, HEPES, 10%v/ FCS, 4 mM L-glutamine, 50 µg/mL gentamicin, 100 µg/mL streptomycin. *C. muridarum* was incubated with a 1/20 dilution of serum for 1 hr at 37°C with 5% CO_2, then added to RAW264.7 cells at an MOI of 1 and incubated for 2, 4, 6 and 24 hrs.

Intrapenile and intravaginal infections

Intrapenile challenges were performed on mice by exposing the glans and inoculating the exterior of the urethra with $1x10^6$ IFU of *C. muridarum* in 5 µL of sucrose-phosphate-glutamine (SPG) (219 mM sucrose, 3.8 mM KH_2PO_4, 8.6 mM Na_2HPO_4, 4.9 mM glutamic acid, pH 7.35).

Female mice received 2.5 mg of medroxyprogesterone (DepoProvera) subcutaneously, seven days prior to challenge with *C. muridarum*. Mice anaesthetized with ketamine (Parnell Laboratory, Alexandria, Australia) (100 mg/Kg) /xylazine (Bayer, Leverkusen, Germany) (10 mg/Kg) were challenged intravaginally with *C. muridarum* in 20 µL of SPG two weeks following immunization. Mice recovering from a primary infection of $5x10^1$, $1x10^2$ or $5x10^2$

IFU (6 weeks) were also re-challenged with the same dose as the primary infection to act as a live infection control (LIC) group.

Prostatic fluid collection, mating and sexual transmission

Male mice were maintained under isoflurane-induced anesthesia and placed on their backs for collecting prostatic fluid. Urine was drained from the bladder using a syringe prior to collection. Ejaculation was induced following electro-stimulation of the prostate using a custom made probe and the square wave generator (Universal Kymograph – Harvard Apparatus, Massachusetts, USA) (Supplementary Figure 6). The prostatic fluid was collected into a capillary tube and expelled into a tube before making the volume up to 20 µL with cold SPG.

Infected males were mated with non-progesterone primed females during the peak of their infection (day 7 – 14 post-infection – p.i) at the beginning of the 12 hr dark cycle. Paired mice were observed for 1 hr and vaginal lavages were collected following the first instances in which a female mated with a male for ≥10 sec. Paired mice were then left for the remaining dark cycle and inspected for vaginal plugs at the beginning of the following 12 hr light cycle. Vaginal plugs were excised and homogenized in 800 µL of SPG using the OMNI TH tissue homogenizer. The uterine horns were flushed with 200 µL of the same SPG containing the vaginal plug.

Quantification of C. muridarum *from secretions, tissues and swabs*

Infected tissues (≤250 mg) were excised and homogenized in 800 µL of SPG. Vaginal swabs were collected using a sterile nasopharyngeal swab (Copan, Murrieta, USA) and stored in 500 µL of SPG containing two glass beads. The SPG supernatant was used to quantify chlamydial content by culture as previously described (21). Ejaculate was also screened by culture and *omp*A-specific qRT-PCR as described previously (31).

RNA extraction and RT-PCR

Genital tract tissues were equilibrated in RNAlater (QIAGEN, Venlo, Netherlands) then total RNA was extract using the RNeasy Tissue Mini Kit (Cat. No. 74704) and treated with RNase-free DNase (QIAGEN). RNA was extracted immediately from sorted cells using Trizol (Invitrogen) and 10 µg of glycogen carrier protein (Cat No. AM9510, Applied Biosystems, Foster City, USA) according to manufacturer's instructions. cDNA was synthesized using High Capacity Reverse Transcriptase Kit (Applied Biosystems) (Cat. No. 4368814) as per the manufacturer's instructions. Each reaction contained 10 ng of cDNA, 1 µM of forward/reverse primers (Supplementary Figure 7) (Sigma-Aldrich), 200 µM dNTP's, 1.5 mM $MgCl_2$, 1X buffer, 0.15X SYBR green, and 5 U of Platinum Taq polymerase (Invitrogen) made up to a final 20µL volume using sterile endonuclease-free water. RT-PCR was performed using the Corbet Rotorgene Q (QIAGEN). Gene expression was analyzed using Hierarchical Clustering Explorer 3.5 Analysis Tool (University of Maryland, USA).

Immunohistochemistry

Immunohistochemistry was performed by the HistoTechnology Facility (Queensland Institute of Medical Research, Brisbane, Australia) as described previously (21).

Gross oviduct pathology

The oviduct diameter was measured on day 35 p.i to assess the severity of pathology (33, 34). The incidence of oviduct pathology (presence or absence) was the number of mice presenting with hydrosalpinx (unilateral and bilateral).

Power calculations and statistical analysis

Sample sizes were determined *a priori* using a one-tailed, Proportions: Inequality, two independent groups Fischer's exact test in G*Power 3.1.7 software (Institute for Experimental Psychology, Dusseldorf, Germany). Statistical analysis of graphical data was performed using GraphPad Prism® version 5.00 (GraphPad, La Jolla, USA).

References

1. ABS. Year book Australia 2012 2012. Available from: http://www.abs.gov.au/ausstats/abs@.nsf/Lookup/by Subject/1301.0~2012~Main Features~Communicable diseases~232.
2. Gerbase AC, Rowley JT, Heymann DH, Berkley SF, Piot P. Global prevalence and incidence estimates of selected curable STDs. Sex Transm Infect. 1998 Jun;74 Suppl 1:S12-6. PubMed PMID: 10023347. Epub 1999/02/19. eng.
3. Stamm WE, Holmes KK. Chlamydia trachomatis infections of the adult. 2nd ed. Holmes KK, Sparling PF, Stamm WE, Piot P, Wasserheit JN, Corey L, editors. New York: : McGraw-Hill; 1990.
4. Patel R, Simms I, Robinson A, LaMontagne S, Van Der Pol B, Moss T, et al. International Handbook of Chlamydia. 3rd ed. Moss TR, editor. Haslemere: Alden Press; 2008. 205 p.
5. Gray RT, Beagley KW, Timms P, Wilson DP. Modeling the Impact of Potential Vaccines on Epidemics of Sexually Transmitted Chlamydia trachomatis Infection. J Infect Dis. 2009 Jun 1;199(11):1680-8. PubMed PMID: 19432550. Epub 2009/05/13. eng.
6. Tuffrey M, Taylor-Robinson D. Progesterone as a key factor in the development of a mouse model for genital-tract infection with Chlamydia trachomatis. FEMS Microbiology Letters. 1981;12(2):111-5.
7. Coleman DL, Kaliss N, Dagg CP, Russell ES, Fuller JL, Staats S, et al. Biology of the Laboratory Mouse. 2 ed. Green EL, editor. NEW YORK: DOVER PUBLICATIONS, INC.; 1968. 706 p.
8. Carey AJ, Cunningham KA, Hafner LM, Timms P, Beagley KW. Effects of inoculating dose on the kinetics of Chlamydia muridarum genital infection in female mice. Immunol Cell Biol. 2009 May-Jun;87(4):337-43. PubMed PMID: 19204735. Epub 2009/02/11. eng.
9. Maxion HK, Liu W, Chang MH, Kelly KA. The infecting dose of Chlamydia muridarum modulates the innate immune response and ascending infection. Infect Immun. 2004 Nov;72(11):6330-40. PubMed PMID: 15501762. Epub 2004/10/27. eng.
10. Al-Mously N, Cross NA, Eley A, Pacey AA. Real-time polymerase chain reaction shows that density centrifugation does not always remove Chlamydia trachomatis from human semen. Fertil Steril. 2009 Nov;92(5):1606-15. PubMed PMID: 18990376. Epub 2008/11/08. eng.

11. Mount DT, Bigazzi PE, Barron AL. Experimental genital infection of male guinea pigs with the agent of guinea pig inclusion conjunctivitis and transmission to females. Infect Immun. 1973 Dec;8(6):925-30. PubMed PMID: 4594119. Epub 1973/12/01. eng.
12. Rank RG, Bowlin AK, Reed RL, Darville T. Characterization of chlamydial genital infection resulting from sexual transmission from male to female guinea pigs and determination of infectious dose. Infect Immun. 2003 Nov;71(11):6148-54. PubMed PMID: 14573630. Epub 2003/10/24. eng.
13. Robertson SA, Guerin LR, Bromfield JJ, Branson KM, Ahlstrom AC, Care AS. Seminal fluid drives expansion of the CD4+CD25+ T regulatory cell pool and induces tolerance to paternal alloantigens in mice. Biol Reprod. 2009 May;80(5):1036-45. PubMed PMID: 19164169. Pubmed Central PMCID: 2849830.
14. Farris CM, Morrison RP. Vaccination against Chlamydia genital infection utilizing the murine C. muridarum model. Infect Immun. 2011 Mar;79(3):986-96. PubMed PMID: 21078844. Epub 2010/11/17. eng.
15. Morelli AB, Becher D, Koernig S, Silva A, Drane D, Maraskovsky E. ISCOMATRIX: a novel adjuvant for use in prophylactic and therapeutic vaccines against infectious diseases. J Med Microbiol. 2012 Jul;61(Pt 7):935-43. PubMed PMID: 22442293. Epub 2012/03/24. eng.
16. O'Meara CP, Andrew DW, Beagley KW. The mouse model of Chlamydia genital tract infection: a review of infection, disease, immunity and vaccine development. Curr Mol Med. 2013 Mar;14(3):396-421. PubMed PMID: 24102506. Epub 2013/10/10. eng.
17. Farris CM, Morrison SG, Morrison RP. CD4+ T cells and antibody are required for optimal major outer membrane protein vaccine-induced immunity to Chlamydia muridarum genital infection. Infect Immun. 2010 Oct;78(10):4374-83. PubMed PMID: 20660610. Epub 2010/07/28. eng.
18. Gebhardt T, Wakim LM, Eidsmo L, Reading PC, Heath WR, Carbone FR. Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. Nat Immunol. 2009 May;10(5):524-30. PubMed PMID: 19305395.
19. Olsen AW, Follmann F, Erneholm K, Rosenkrands I, Andersen P. Protection Against Chlamydia trachomatis Infection and Upper Genital Tract Pathological Changes by Vaccine-Promoted Neutralizing Antibodies Directed to the VD4 of the Major Outer Membrane Protein. The Journal of infectious diseases. 2015 Sep 15;212(6):978-89. PubMed PMID: 25748320.
20. Kobzik L, Huang S, Paulauskis JD, Godleski JJ. Particle opsonization and lung macrophage cytokine response. In vitro and in vivo analysis. J Immunol. 1993 Sep 1;151(5):2753-9. PubMed PMID: 8360489. Epub 1993/09/01. eng.
21. O'Meara CP, Armitage CW, Harvie MC, Andrew DW, Timms P, Lycke NY, et al. Immunity against a Chlamydia infection and disease may be determined by a balance of IL-17 signaling. Immunol Cell Biol. 2013 Mar;92(3):287-97. PubMed PMID: 24366518. Epub 2013/12/25. eng.
22. Mueller SN, Gebhardt T, Carbone FR, Heath WR. Memory T cell subsets, migration patterns, and tissue residence. Annu Rev Immunol. 2012;31:137-61. PubMed PMID: 23215646. Epub 2012/12/12. eng.

23. Schenkel JM, Fraser KA, Vezys V, Masopust D. Sensing and alarm function of resident memory CD8(+) T cells. Nat Immunol. 2013 May;14(5):509-13. PubMed PMID: 23542740. Epub 2013/04/02. eng.
24. Laidlaw BJ, Zhang N, Marshall HD, Staron MM, Guan T, Hu Y, et al. CD4+ T cell help guides formation of CD103+ lung-resident memory CD8+ T cells during influenza viral infection. Immunity. 2014 Oct 16;41(4):633-45. PubMed PMID: 25308332. Pubmed Central PMCID: 4324721.
25. Shin H, Iwasaki A. A vaccine strategy that protects against genital herpes by establishing local memory T cells. Nature. 2012;491(7424):463-7.
26. Craig AP, Hanger J, Loader J, Ellis WA, Callaghan J, Dexter C, et al. A 5-year Chlamydia vaccination programme could reverse disease-related koala population decline: Predictions from a mathematical model using field data. Vaccine. 2014 Jul 16;32(33):4163-70. PubMed PMID: 24877768. Epub 2014/06/01. eng.
27. Fijak M, Meinhardt A. The testis in immune privilege. Immunol Rev. 2006 Oct;213:66-81. PubMed PMID: 16972897. Epub 2006/09/16. eng.
28. Armitage CW, O'Meara CP, Harvie MC, Timms P, Blumberg RS, Beagley KW. Divergent outcomes following transcytosis of IgG targeting intracellular and extracellular chlamydial antigens. Immunol Cell Biol. 2013 Jan 21:. In print. PubMed PMID: 24445600. Epub 2014/01/22. Eng.
29. Mayhew A, Mullins TL, Ding L, Rosenthal SL, Zimet GD, Morrow C, et al. Risk perceptions and subsequent sexual behaviors after HPV vaccination in adolescents. Pediatrics. 2014 Mar;133(3):404-11. PubMed PMID: 24488747. Epub 2014/02/04. eng.
30. Caldwell HD, Kromhout J, Schachter J. Purification and partial characterization of the major outer membrane protein of Chlamydia trachomatis. Infect Immun. 1981 Mar;31(3):1161-76. PubMed PMID: 7228399. Epub 1981/03/01. eng.
31. O'Meara CP, Armitage CW, Harvie MCG, Timms P, Lycke NY, Beagley KW. Immunization with a MOMP-Based Vaccine Protects Mice against a Pulmonary Chlamydia Challenge and Identifies a Disconnection between Infection and Pathology. PLoS One. 2013;8(4):e61962.
32. Smyth MJ, Thia KY, Street SE, MacGregor D, Godfrey DI, Trapani JA. Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. J Exp Med. 2000 Sep 4;192(5):755-60. PubMed PMID: 10974040. Pubmed Central PMCID: 2193269.
33. Shah AA, Schripsema JH, Imtiaz MT, Sigar IM, Kasimos J, Matos PG, et al. Histopathologic changes related to fibrotic oviduct occlusion after genital tract infection of mice with Chlamydia muridarum. Sex Transm Dis. 2005 Jan;32(1):49-56. PubMed PMID: 15614121. Epub 2004/12/23. eng.
34. Imtiaz MT, Schripsema JH, Sigar IM, Kasimos JN, Ramsey KH. Inhibition of matrix metalloproteinases protects mice from ascending infection and chronic disease manifestations resulting from urogenital Chlamydia muridarum infection. Infect Immun. 2006 Oct;74(10):5513-21. PubMed PMID: 16988226. Epub 2006/09/22. eng.

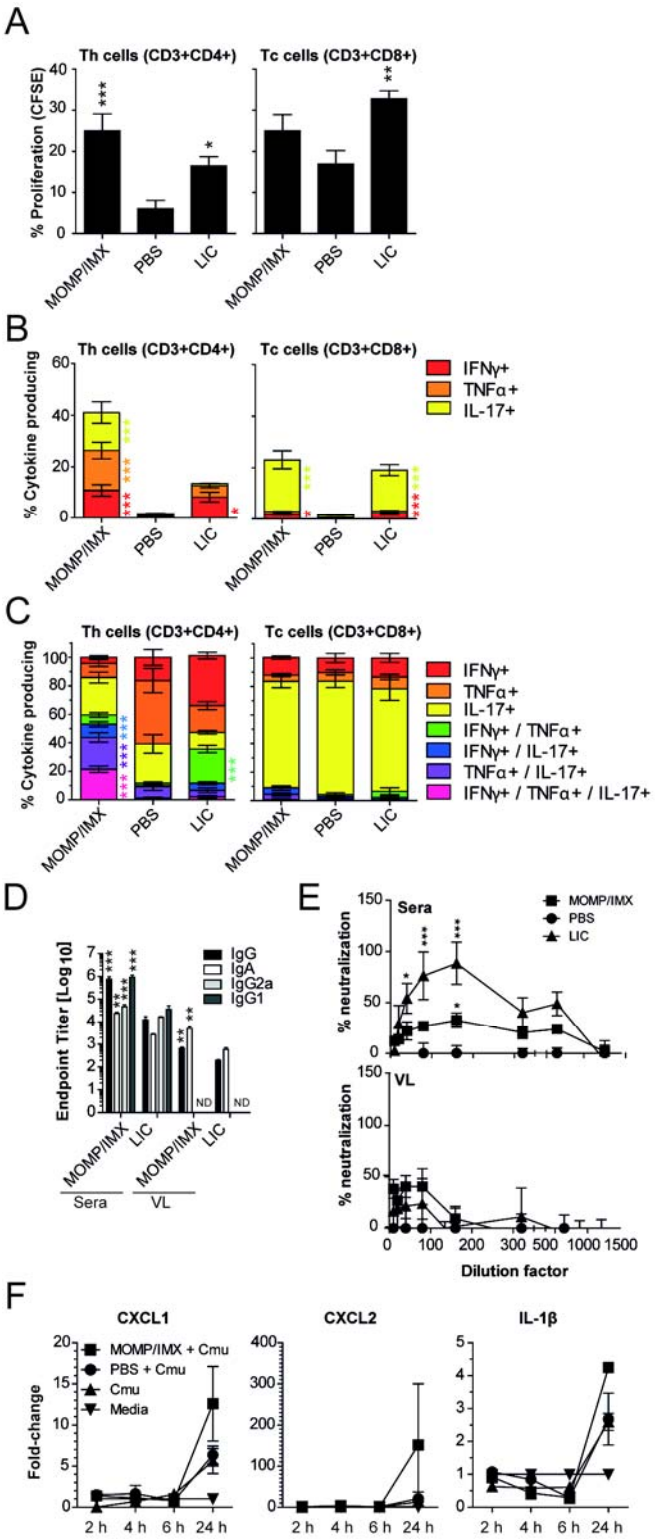


Figure 1: MOMP-specific T and B cell responses following immunization

Cells were isolated from spleens of immunized (MOMP/IMX) or nonimmunized (PBS) mice (7 day post-immunization) or infected mice (intravaginally challenged with $5x10^4$ IFU of *C. muridarum*, 21 day p.i), stimulated with MOMP for 5 days and analyzed by flow cytometry for antigen-specific proliferation and cytokine production. Live cells (viability stain) were gated initially on single cells (FSC-A/FSC-H) and lymphocytes (FSC/SSC) before gating CD3+ cells on Th (CD3+CD4+) and Tc cells (CD3+CD8+). (A) Cells were labeled with

CFSE and stimulated with MOMP to measure the percentages of MOMP-specific Th and Tc cell proliferation (n = 10). (B) MOMP-specific Th cells staining positive for IFNγ, TNFα and IL-17 are represented as a percentage of total Th cells (n = 10). (C) MOMP-specific Th cells staining positive for TNFα were assessed for multifunctional phenotype (IFNγ and IL-17) are represented as a percentage of total cytokine producing Th cells (n = 10). (D) MOMP-specific IgG, IgA, IgG2a and IgG1 endpoint titers were quantified in serum and vaginal lavage (VL) samples by ELISA. (E) *In vitro* neutralization of *Chlamydia* infectivity was determined for serum and vaginal lavage by culture (n = 10). (F) The ability of serum collected from MOMP/IMX immunized mice to opsonize *Chlamydia* and promote CXCL1, CXCL2 and IL-1β production by monocytes/macrophages (RAW264.7 cell line) was determined using RT-PCR. Results are presented as the mean ± SEM. ND refers to "not done". 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 (***).

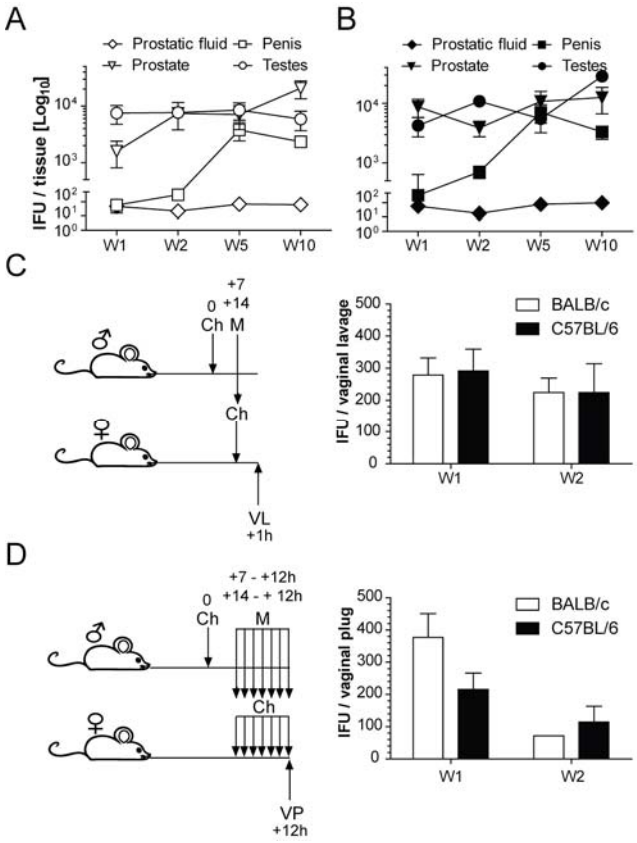


Figure 2: Quantification of the chlamydial dose sexually transmitted by male mice.

(A) Male BALB/c and (B) C57BL/6 mice were challenged with $1x10^6$ IFU *C. muridarum* and the chlamydial burden was quantified from the prostatic fluid, penis, prostate and testes collected on week 1, 2, 5 and 10 p.i by culture (n = 5). (C) Male mice were challenged (Ch) with *C. muridarum* and mated (M) with non-progesterone primed females (receptive) and the chlamydial load transmitted was determined from vaginal lavages (VL) (n = 11) and (D) vaginal plugs (VP) (n = 15) during weeks 1 and 2 of their infection. Results are presented, as the mean ± SEM. 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 (***).

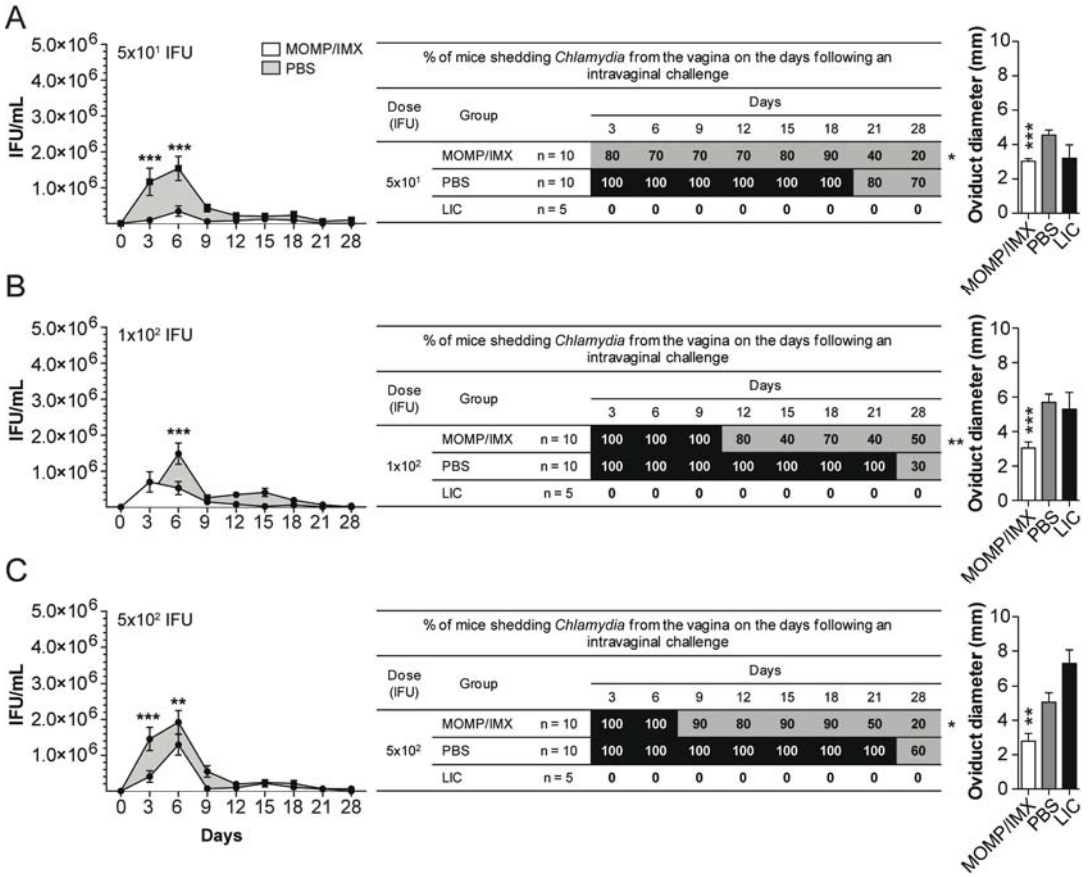


Figure 3: Protection against genital tract infection and oviduct pathology in female mice following immunization and challenge with sexually transmissible doses of *C. muridarum*.

Female BALB/c mice were immunized (MOMP/IMX), nonimmunized (PBS) or infected (1° infection, intravaginally challenged with $5x10^1$, $1x10^2$ and $5x10^2$ IFU of *C. muridarum*) then challenged with (A) $5x10^1$, (B) $1x10^2$, (C) $5x10^2$ IFU of *C. muridarum*. Vaginal swabs were collected over 28 days p.i to quantify the amount of *Chlamydia* shed (IFU/mL) by culture. Significant levels of bacterial burden were determined using a two -way ANOVA with Tukey's post -test. Results are presented as the mean ± SEM. Percentages of mice shedding *Chlamydia* from the vagina on the days following challenge are also presented. Significant differences were determined using a Kaplan -Meier survival curve and the log rank post -test. The severity of pathology was determined by measuring the diameter of the oviducts post-mortem on day 35 p.i. The solid line across the graph represents the mean width of unaffected oviducts (2 mm). Significance of disease severity was 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 (***).

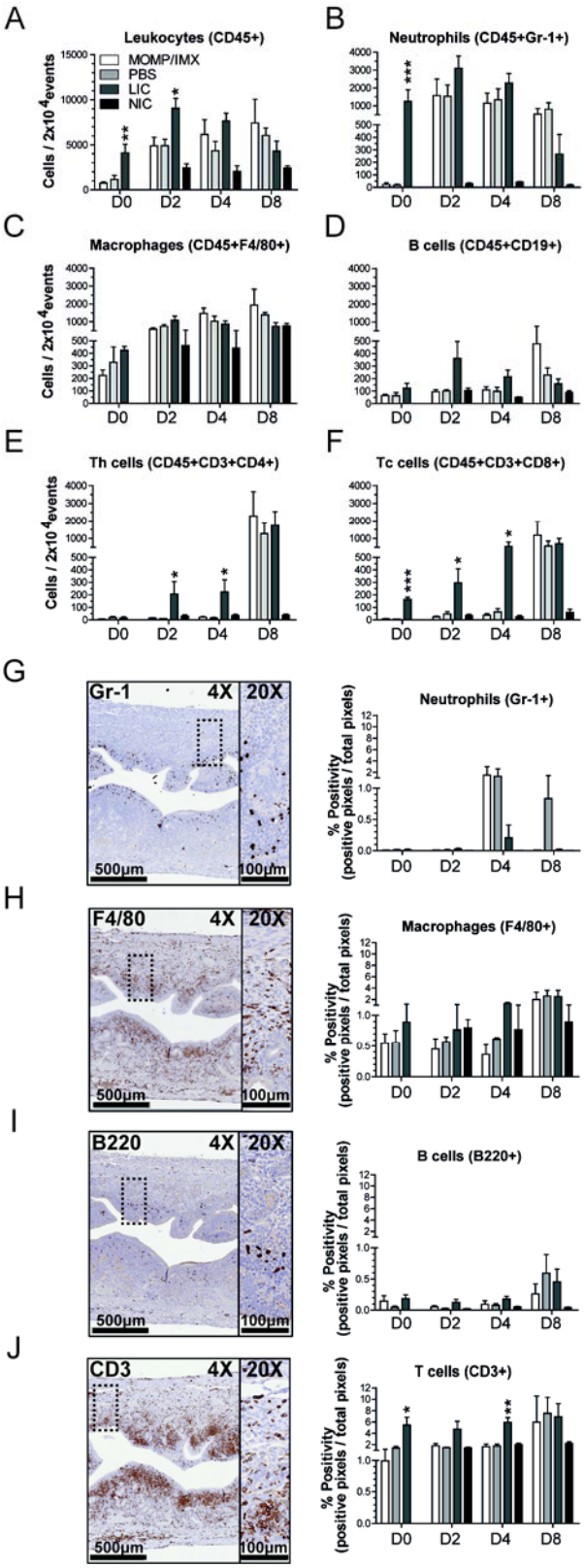


Figure 4: Kinetics of leukocyte, neutrophil, macrophage, B, Th and Tc cell recruitment following immunization and challenge with a sexually transmissible dose of *C. muridarum*.

Female BALB/c mice were immunized (MOMP/IMX), nonimmunized (PBS) or infected (1° infection, intravaginally challenged with $5x10^1$ IFU of *C. muridarum*) then challenged with

687 $5x10^1$ IFU of *C. muridarum*. Tissues were collected from immunized mice (MOMP/IMX and
688 PBS) prior to challenge (day 0) and on days 2, 4 and 8 following challenge (n = 6). Tissues
689 were also collected from uninfected animals (no infection control – NIC) on days 2, 4 and 8
690 and mice recovering from an intravaginal infection (day 0 = 1° infection, day 21 p.i) and on
691 days 2, 4 and 8 p.i following re-challenge (live infection control – LIC) (n = 6). Cells were
692 isolated from the uterine horns and cervix/vagina by digestion for analysis by flow cytometry.
693 Live cells (viability stain) were gated initially on single cells (FSC-A/FSC-H) and (A)
694 leukocytes (CD45+) before gating on (B) neutrophils (Gr-1+), (C) macrophages (F4/80+),
695 (D) B cells (CD19+), (E) Th cells (CD3+CD4+) and (F) Tc cells (CD3+CD8+). Results are
696 presented as number of cells per $2x10^4$ events. Uterine horns were also fixed for analysis by
697 immunohistochemistry (n = 6). Serial tissue sections of the uterine horns were stained for the
698 presence of (G) neutrophils (Gr-1+), (H) macrophages (F4/80+), (I) B cells (B220+), (J) and
699 T cells (CD3+). Representative images (4x and 20x magnifications) are shown for each stain.
700 ImageScope software was used to quantify the percentage positivity (positive pixels/total
701 pixels) from a high-resolution image of each section. Results are presented as the mean ±
702 SEM. Significant differences were determined using a one-way ANOVA with Tukey's post-
703 test. Significance was set at $p < 0.05$ for all tests. $p>0.05$ (not shown), 0.01-0.05 (*), 0.001-
704 0.01 (**) and <0.001 (***).
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719

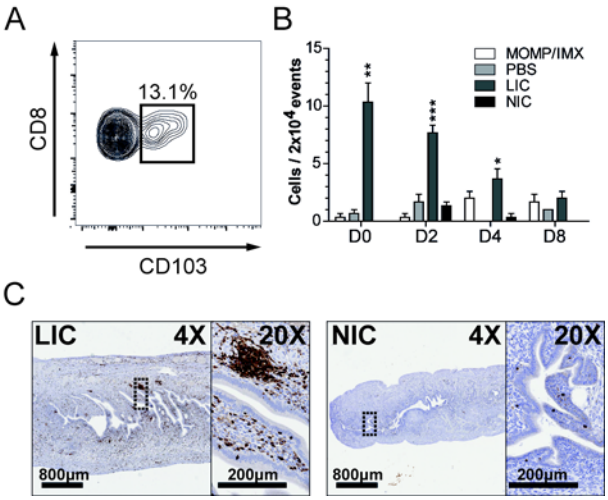


Figure 5: Kinetics of T_{RM} cells (CD3+CD8+CD103+) following immunization and challenge with a sexually transmissible dose of *C. muridarum*.

Female BALB/c mice were immunized (MOMP/IMX), nonimmunized (PBS) or infected (1° infection, intravaginally challenged with $5x10^1$ IFU of *C. muridarum*) then challenged with $5x10^1$ IFU of *C. muridarum*. Tissues were collected from immunized mice (MOMP/IMX and PBS) prior to challenge (day 0) and on days 2, 4 and 8 following challenge (n = 3). Tissues were also collected from uninfected animals (no infection control – NIC) and mice recovering from an intravaginal infection (day 0 = 1° infection, day 21 p.i) and on days 2, 4 and 8 p.i following re-challenge (live infection control – LIC) (n = 3). Cells were isolated from the uterine horns and cervix/vagina by digestion for analysis by flow cytometry. (A) Resident memory Tc were identified by gating on lymphocytes (FSC/SSC), single cells (FSC -A/FSC -H), leukocytes (CD45+), Tc cells (CD3+CD8+) and finally CD103+. (B) Cells per $2x10^4$ events are presented for each group. Results are presented as the mean ± SEM. (C) CD3 staining of tissue sections from uterine horns collected from LIC and NIC groups prior to challenge. 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 (***).

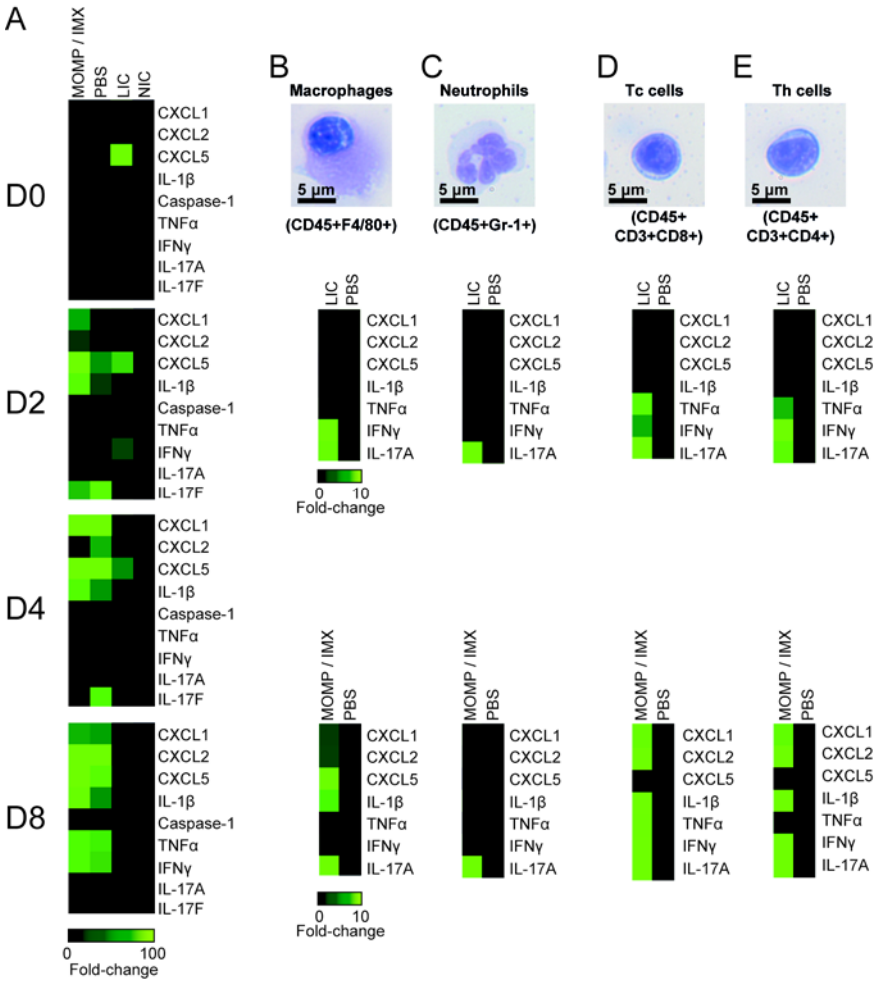


Figure 6: Gene expression in whole oviducts, macrophages, neutrophils, Tc and Th cells isolated from the oviducts following immunization and challenge with a sexually transmissible dose of *C. muridarum*.

Female BALB/c mice were immunized (MOMP/IMX), nonimmunized (PBS) or infected (1° infection, intravaginally challenged with $5x10^1$ IFU of *C. muridarum*) then challenged with $5x10^1$ IFU of *C. muridarum*. Tissues were collected from immunized mice (MOMP/IMX and PBS) prior to challenge (day 0) and on days 2, 4 and 8 following challenge. Tissues were also collected from uninfected animals (no infection control – NIC) and mice recovering from an intravaginal infection (day 0 = 1° infection, day 21 p.i) and on days 2, 4 and 8 p.i following re-challenge (live infection control – LIC) (n = 3). (A) Gene expression was performed on pooled oviducts (n = 3) and fold change (scale 0 – 100-fold) was calculated relative to the uninfected control (NIC). Live cells were isolated from the genital tract on days 2 and 8 p.i and separated into (B) macrophages (CD45+F4/80+) (C) neutrophils (CD45+Gr-1+), (D) Tc (CD45+CD3+CD8+) and (E) Th cells (CD45+CD3+CD4+) using flow cytometry (n = 10). Cytospin and Geimsa staining was performed on sorted cell fractions to compare surface marker phenotype with nuclear morphology (representative image shown). Gene expression was performed on pooled cells isolated from the oviducts, uterine horns and cervix vagina (n = 10), and fold changes (scale 0 – 10-fold) were calculated relative to an unimmunized control. 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 (***).

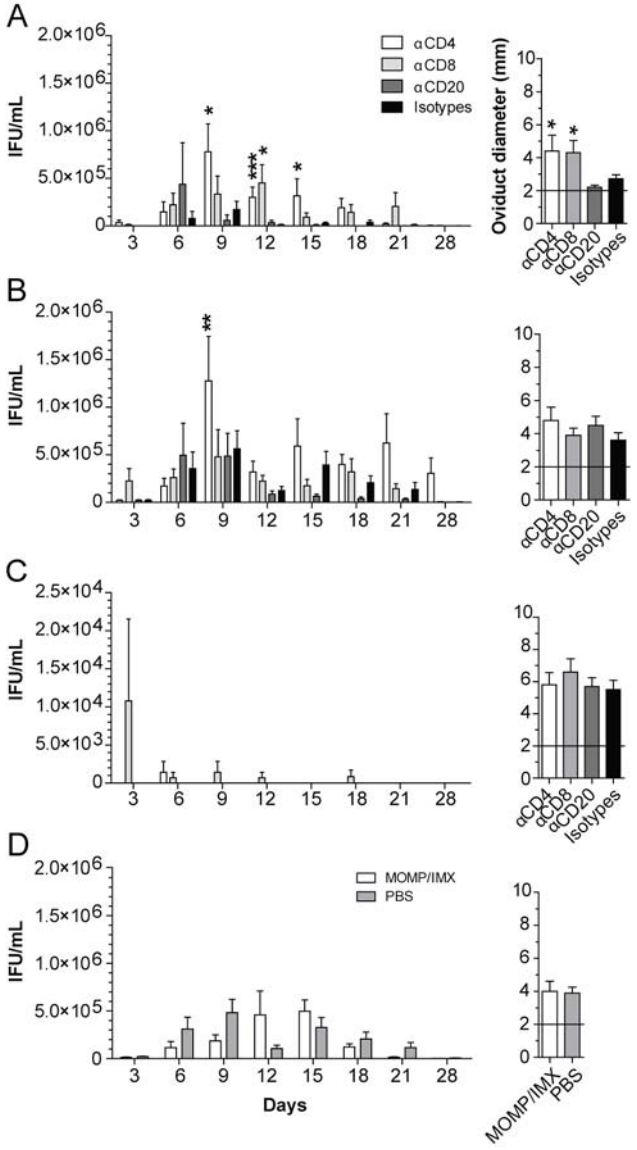


Figure 7: *In vivo* depletion (CD4+, CD8+ and CD20+) and passive immunization prior to challenge with a sexually transmissible dose of *C. muridarum*.

Female BALB/c mice were immunized (MOMP/IMX), nonimmunized (PBS) or infected (1° infection, intravaginally challenged with $5x10^4$ IFU of *C. muridarum*) and depleted of CD4, CD8 and CD20 cells (n = 5). Unimmunized mice were also passively immunized with serum collected from MOMP/IMX immunized mice (n = 5). Depleted and passively immunized mice were then challenged with $5x10^1$ IFU of *C. muridarum*. Vaginal infection and oviduct pathology were quantified from (A) MOMP/IMX, (B) unimmunized, (C) LIC and (D) passively immunized naïve mice. Vaginal swabs were collected for 28 days p.i to quantify the amount of *Chlamydia* shed (IFU/mL) by culture. The severity of pathology was determined on day 35 p.i by measuring the diameter of the oviducts. The solid line across the graph represents the mean width of unaffected oviducts (2 mm). Results are presented as the mean ± SEM. Significant levels of bacterial burden and disease severity 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 (***).

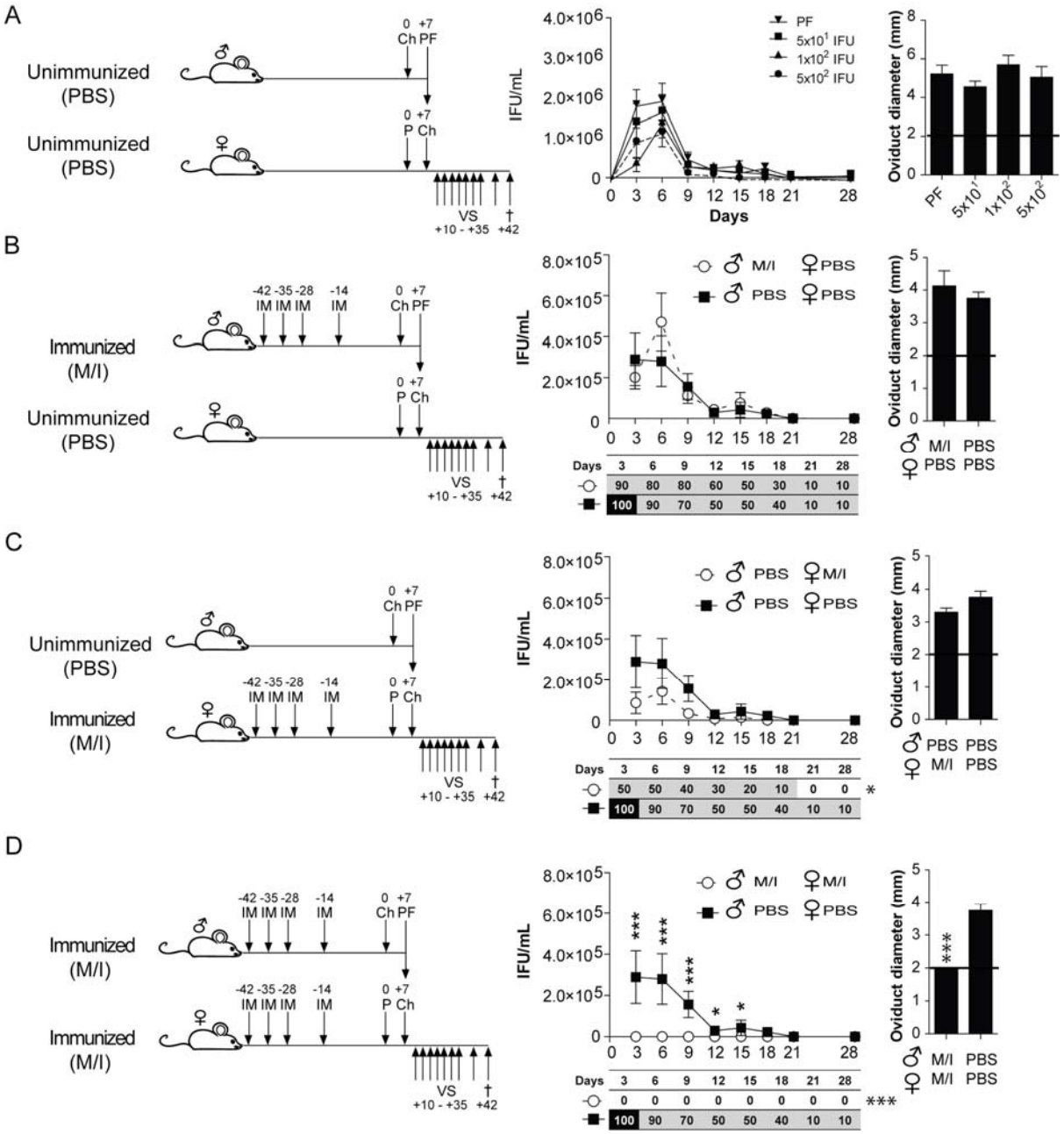


Figure 8: Transmission of *Chlamydia* between male and female mice with different vaccination statuses.

(A) Male mice were challenged (Ch) with $1x10^6$ IFU *C. muridarum* and their prostatic fluids (PF) were collected 7 days p.i (n = 10). Female mice were progesterone-primed (P) 7 days prior to being challenged intravaginally with prostatic fluid collected from the infected males (n = 5). Vaginal shedding and development of hydrosalpinx following challenge with PF was compared to challenge of female mice with $5x10^1$, $1x10^2$ and $5x10^2$ of *C. muridarum*. This model of sexual transmission was repeated using (B) males, (C) females and (D) males and females that had been immunized (IM) with MOMP/IMX (M/I) prior to challenge with PF (n = 10). Vaginal swabs (VS) were collected over 28 days p.i to quantify the amount of *Chlamydia* shed (IFU/mL) by culture. Significant levels of bacterial burden were determined using a two-way ANOVA with Tukey's post-test. Results are presented as the mean ± SEM. Percentages of mice shedding *Chlamydia* from the vagina on the days following challenge

are also presented. Significant differences were determined using a Kaplan -Meier survival curve and the log rank post-test. Protection against the severity of oviduct pathology (hydrosalpinx) in female mice challenge with prostatic fluid was determined post mortem (†) on day 35 p.i by measuring oviduct diameter. The solid line across the graph represents the mean width of unaffected oviducts (2 mm). Results are presented as the mean ± SEM. Significant levels of bacterial burden and disease severity 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 (***).

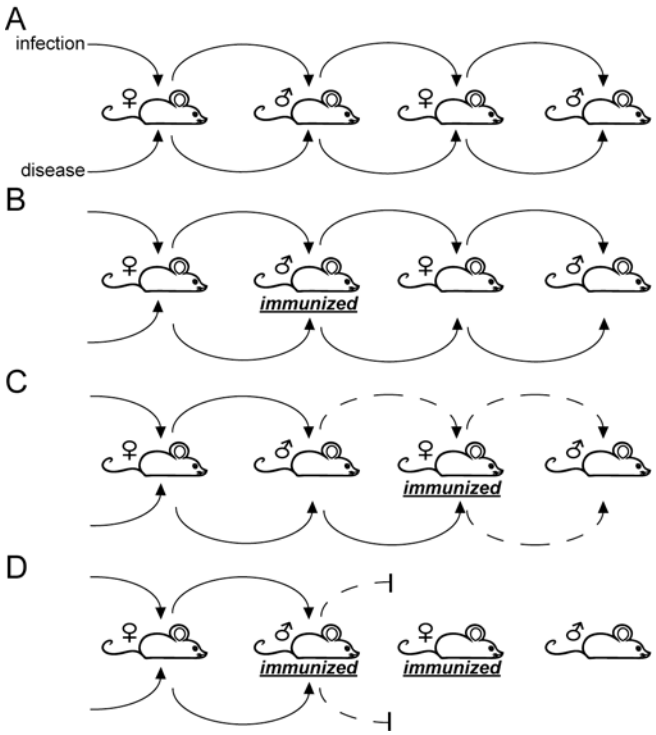


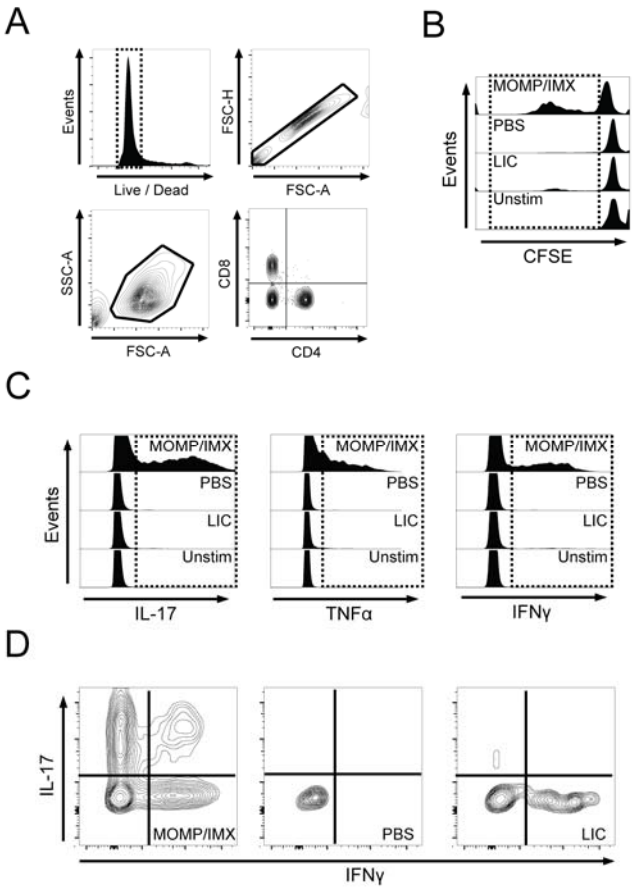
Figure 9: Prevention of *Chlamydia* sexual transmission by vaccination.

(A) *Chlamydia* infection transmission occurs unimpeded though unimmunized mice. (B) Risk of infection transmission to unimmunized females is unaffected by the male vaccination status. (C) Vaccinating female mice reduces their risk of acquiring an infection from unimmunized males by 50%, but not their risk of developing disease. As half of immunized females were protected from an infection, risk of infection transmission and disease developing in unimmunized males is also reduced. (D) No transmission of infection occurs between immunized males and females. Immunized females show no signs of disease. Further transmission to unimmunized males is also prevented.

S1: *Chlamydia* quantified from infectious male mouse ejaculate

Sample	Prostatic fluid	Vaginal lavage	Vaginal plug
Min – Max (IFU)	18 – 224	135 – 449	72 – 503
Mean ± SD (IFU)	68 ± 45	261 ± 98	220 ± 150
Min – Max (*omp*A copies)	$2080 – 1.2x10^5$	ND	$1.7x10^5 – 3x10^7$
Mean ± SD (*omp*A copies)	$3x10^4 ± 665^7$	ND	$5x10^6 ± 8.5x10^6$

Minimum, maximum and mean amount of *Chlamydia* collected from prostatic fluid, vaginal lavage and vaginal plugs determined by culture (IFU) and RT-PCR (*omp*A copies). Results are a combination of both strains (BALB/c and C57BL/6) and time points (W1 and W2). ND abbreviation refers to "not done".



865

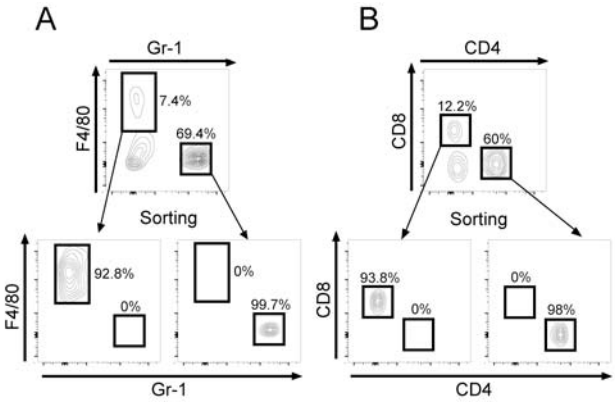
866 **S2: Gating strategies for MOMP-specific T cell responses following immunization**

867 (A) Live cells (viability stain) were gated initially on single cells (FSC-A/FSC-H) and
868 lymphocytes (FSC/SSC) before gating CD3+ cells on Th (CD3+CD4+) and Tc cells
869 (CD3+CD8+). (B) Cells were labeled with CFSE and stimulated with MOMP to measure the
870 percentages of MOMP-specific Th and Tc cell proliferation. (C) MOMP-specific Th cells
871 staining for IFNγ, TNFα and IL-17. (D) MOMP-specific Th cells staining positive for TNFα
872 were assessed for multifunctional phenotype (IFNγ and IL-17) and represented as a
873 percentage of total cytokine producing Th cells.

874

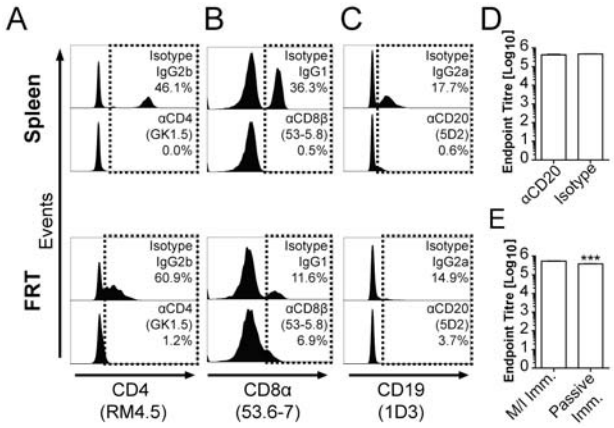
875

876



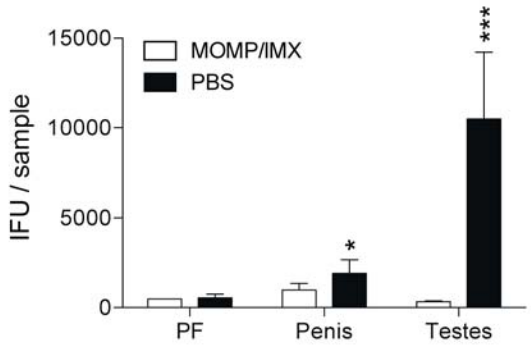
S3: Gating strategies for macrophages, neutrophils, Tc and Th cells isolation and purity

Live cells were isolated from the genital tract on days 2 and 8 p.i and separated into (A) macrophages (CD45+F4/80+) and neutrophils (CD45+Gr-1+), (B) Tc (CD45+CD3+CD8+) and Th cells (CD45+CD3+CD4+) using flow cytometry. Representative plots for macrophages (92.8%) and neutrophils (99.7%), Tc (93.8%) and Th cells (98%) purity are shown.



S4: Confirmation of *in vivo* depletion (CD4+, CD8+ and CD20+) and passive immunization

Spleens and genital tracts were collected 2 days following intraperitoneal infusion of (A) αCD4, (B) αCD8 and (C) αCD20 and depletion was confirmed using flow cytometry. Depletion was compared against isotype control infused mice. (D) Serum was screened for the presence of MOMP-specific Ig in MOMP/IMX immunized mice by ELISA following αCD20 infusion. (E) MOMP-specific Ig in the serum collected from MOMP/IMX immunized donor mice (M/I Imm.) and serum from recipient naïve mice passively immunized with MOMP/IMX serum (Passive Imm.) were analyzed by ELISA.



S5: Protection against genital tract infection in male mice following immunization and challenged with *C. muridarum*.

Male mice were challenged with $1x10^6$ IFU *C. muridarum* and their prostatic fluid (PF), penis and testes were collected 7 days p.i (n = 5). The amount of *Chlamydia* presented in each sample (IFU/sample) was quantified by culture. Results are presented as the mean ± SEM. Significant levels of bacterial burden and disease severity 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 (***).

S6: Electro-stimulation schedule for collection of prostatic fluid

	Frequency (Hz)	Pulse width (ms)	Intensity (V)	Duration (min:sec)
*1.	1.0	1.0	0.5	0:30
2.	1.0	1.0	1.0	0:30
3.	1.0	1.0	1.5	0:30
4.	1.0	1.0	2.0	0:30
5.	2.0	1.0	2.5	0:30
6.	2.0	1.0	5.0	0:30
#7.	2.0	1.0	7.5	0:30
8.	2.0	1.0	10.0	0:30
†9.	2.0 – 10.0 – 2.0	1.0	10.0	0:04 x 5
10.	2.0 – 10.0 – 2.0	1.0	15.0	0:04 x 5
11.	2.0 – 10.0 – 2.0	1.0	20.0	0:04 x 5
			Total	5:00

*Urine contamination can be produced early during the procedure and must be discarded. #Prostatic fluid is typical produced from this point onwards. †Frequency is increased for 1 sec, held at maximum for 2 sec and decreased for 1 sec. This cycle is repeated five times for each intensity setting

977 **S7: Primers for RT-PCR**

Gene	Accession No.	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
CXCL1	NM_008176.3	ACCCAAACCGAAGTCATAGCC	TTGTCAGAAGCCAGCGTTCA
CXCL2	NM_009140.2	CCCAGACAGAAGTCATAGCCAC	CGAGGCACATCAGGTACGAT
CXCL5	NM_009141.3	GCCCCTTCCTCAGTCATAGC	AGCTTTCTTTTTGTCACTGCCC
IL-1β	NM_008361.3	TGCCACCTTTTGACAGTGATG	AAGGTCCACGGGAAAGACAC
Caspase-1	NM_009807.2	TGAAAGAATTTGCTGCCTGCC	CCTTGTTTCTCTCCACGGCAT
IFNγ	NM_008337.3	AGGAACTGGCAAAAGGATGGT	TCATTGAATGCTTGGCGCTG
IL-17A	NM_010552.3	TGAGTCCAGGGAGAGCTTCA	CATTGCGGTGGAGAGTCCAG
IL-17F	NM_145856.2	CGTGAAACAGCCATGGTCAAG	GGGGGTCTCGAGTGATGTTG
TNFα	NM_013693.3	ACGTCGTAGCAAACCACCAA	ATAGCAAATCGGCTGACGGT
ActB	NM_007393.3	AGAGGGAAATCGTGCGTGAC	CAATCGTGATGACCTGGCCGT

978

979