Short Communication:

**CD4⁺ T cells reduce the tissue burden of
Chlamydia muridarum in male BALB/c mice**

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Introduction

The burden of chlamydial disease is largely considered a female problem.

However, data is becoming increasingly available which indicates that male infection with *Chlamydia trachomatis* can have significant impacts on public health. The prevalence of infection appears similar in both sexes (reviewed in [1]). In males, a role for *Chlamydia* in the development of sequelae including urethritis, epididymitis and orchitis is accepted, and infection has been implicated as an aetiological agent for prostatitis, with chlamydial incidences of up to 39.5% reported [2].

Chlamydial infection of the testes or prostate is implicated in poor sperm quality [3] and infertility, with up to 29.2% of male partners of infertile couples having anti-chlamydial IgA present in their semen [4]. Furthermore, the presence of anti-chlamydial antibodies in males correlates with infertility factors in their female partners, and subsequent reductions in pregnancy rates [5]. Thus, the significance of chlamydial infections in affecting fertility of couples is undeniable.

To date, most research examining the natural immune response to a chlamydial infection, or vaccine studies, have focussed on female animal models. Studies in mice have indicated that IFN- γ is necessary for the clearance of a chlamydial infection in females [6], thus vaccine candidates aim to elicit such a response. However, it is possible that such an immune response may affect the immune privileged nature of the male reproductive tract, possibly contributing to infertility [7].

Patterson and Rank [8] found that male guinea pigs were more resistant to a secondary challenge with *Chlamydia* than females, and therefore proposed that vaccination of males may reduce population spread of infection.

Modelling studies have also suggested that vaccination of both sexes would reduce overall *Chlamydia*-related morbidity; and targeting 100% of one sex would be likely to have a greater impact than targeting 50% of both sexes [9].

With few infection/vaccine studies conducted in males, the type of immunity required to successfully clear an infection is not yet well understood.

Furthermore, it is now becoming evident that humoral immunity is induced differently between the sexes [10,11]. Here we investigate the contribution of antigen-specific CD4⁺ T cells in clearance of a *Chlamydia muridarum* infection in male mice.

Methods, Results and Discussion

Male BALB/c mice were obtained from the Animal Resources Centre (Perth, Australia). Groups of 10 mice were immunised via the intranasal route with either 5µg cholera toxin (CT) alone (List biological Laboratories, Campbell CA, USA) or 5µg cholera toxin in combination with 100µg of recombinant maltose binding protein-major outer membrane protein (MOMP) of *C. muridarum*.

Recombinant protein was prepared as previously described [12]. *C. muridarum* (Weiss variant) was obtained from the ATCC (VR-123).

Immunisation and boosts were performed on days 0, 7, 14 and 28. Sacrifice of all immunised animals was conducted seven days following the final boost; serum and spleens were harvested. Briefly, serum was separated from cardiac puncture blood by centrifugation, then analysed by ELISA as

described previously for quantitation of MOMP-specific IgG1, IgG2a, and IgA [12].

Immunisation of male mice with CT+MOMP resulted in the induction of high titres of IgG1, approximately 70-fold greater than levels of IgG2a, indicative of a Th2-dominant response. MOMP-specific IgA antibodies were also induced by the intranasal immunisation. Pal and colleagues [13] demonstrated that an intrapenile inoculation of C3H/HeN (H2-K1) mice with *Chlamydia muridarum* resulted in high serum titres of IgG2a. This discrepancy is likely due to the different mouse strains examined in the two studies, and differences in the antigens recognised. The significant role of antibodies, specifically IgA, is implicated by the induction of high levels of IgA in the course of a natural chlamydial infection in men [14]. Furthermore, poly-immunoglobulin receptor knockout mice, incapable of transporting IgA into male reproductive tract secretions, have a reduced capacity to clear an infection [15].

To further investigate the immune response induced by intranasal immunisation with CT+MOMP, T cells were harvested from spleens post-immunisation. Half of the processed T cells were examined for cytokine profiles following MOMP stimulation, while the other half were used for adoptive transfer experiments in naïve mice. To examine cytokine responses, 5×10^5 cells were seeded per well of a 96-well culture plate, and treated with 20µg/ml MOMP or media alone. Cells were incubated at 37°C for four days, then supernatants were harvested and analysed by BioPlex multiplex

suspension array system according to the manufacturer's instructions (Bio-rad Laboratories, North Ryde NSW, Australia).

MOMP stimulation of T cells from mice immunised with CT alone resulted in very little cytokine induction, while stimulation of T cells from mice immunised with CT+MOMP, showed a marked increase in a number of cytokines when compared to unstimulated cells. There was an observed increase in the Th1-related cytokine IL-2 (~50-fold), and roughly 150-fold increases in production of Th2-related IL-10, Th1-related IFN- γ , and inflammatory TNF- α . Similarly, others have shown that induction of IFN- γ and TNF- α is seen following an infection in C3H/HeN mice [13]. In female IgA-deficient mice, *C. muridarum* pulmonary infection generates higher levels of IFN- γ and greater lung pathology than controls, suggesting IgA is a negative regulator of inflammation [16]. The potential for these cytokines to affect male reproductive tract function have not yet been examined *in vivo* and will be the subject of future investigations.

Finally, CD4⁺ T cell subsets were enriched from the T cell population using the CD4⁺ T cell Enrichment kit (BD BioSciences, North Ryde NSW, Australia). Groups of five naïve male BALB/c mice were each given an adoptive transfer of 1×10^7 CD4⁺ T cells, either from the CT only, or CT+MOMP group. Adoptive transfer was by the retro-orbital route. One day later, mice were given an intrapenile inoculation of 5×10^4 ifu *C. muridarum* as previously described [15]. Testes and penile urethral tissues were harvested to examine chlamydial load within the tissues at four weeks post-infection, a time of high

tissue chlamydial load and immune responsiveness in male mice [13,15]. Briefly, total DNA was extracted from tissues using the Promega Wizard kit (Promega, Alexandria NSW, Australia). Chlamydial load in tissues was quantified by Real-Time PCR using flanking nucleotide primers specific for *C. muridarum* MOMP (Forward: 5'-gcc gtt ttg ggt tct gct t-3'; Reverse: 5'-cgt caa tca taa ggc ttg gtt ca-3').

The adoptive transfer of MOMP-specific CD4⁺ T cells was able to significantly reduce the chlamydial load in reproductive tissues following an intrapenile infection with *C. muridarum*, when compared to mice receiving control CD4⁺ T cells. Levels of *Chlamydia* in testes were reduced by more than half, while a reduction of over four-fold was observed in the penile urethra. The significance of such a large reduction in tissue chlamydial load is uncertain, but would almost certainly reduce disease transmission.

The adoptive transfer of chlamydial antigen-specific CD4⁺ T cells has previously been demonstrated to reduce chlamydial infection in female mice [17-19]. Interestingly, the adoptive transfer of chlamydial protease-like activity factor (CPAF)-specific CD4⁺ T cells in IFN- γ -deficient mice was sufficient to produce high levels of protective IFN- γ , while also protecting against pathology of the female genital tract [19]. To our knowledge, this is the first study to demonstrate the protective role of chlamydial antigen-specific CD4⁺ T cells in a *C. muridarum* infection of male mice. Future studies will need to examine the role of Th1 versus Th2 induction, and the role of specific cytokines in the development of male pathology, in order to successfully

develop vaccine strategies that will induce a protective immune response whilst also avoiding tissue damage and potential consequences of infertility.

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