CTA1-DD is an effective adjuvant for targeting anti-chlamydial immunity to the genital mucosa

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Abstract

Chlamydia trachomatis is a significant human pathogen with high prevalence and potentially severe disease sequelae including infertility. A successful vaccine will need to effectively target a combination of humoral and cell-mediated immunity to the genital mucosa. Intranasal immunization can effectively target the genital tract, but the potent mucosal toxin-based adjuvants such as cholera toxin (CT) can cause neurological side effects. CTA1-DD is a non-toxic potent mucosal adjuvant which combines the enzymatic properties of CT, with a B cell targeting moiety. This adjuvant is able to enhance a mixture of Th1/Th2 responses in the mucosal surfaces of the gut and respiratory epithelia. Here, we demonstrate that intranasal immunization with CTA1-DD and chlamydial Major Outer Membrane Protein results in the induction of neutralizing systemic and mucosal antibodies, and is able to enhance protection against a live intravaginal challenge with *Chlamydia muridarum*.

Introduction

Chlamydia trachomatis infections are prevalent worldwide and are responsible for severe disease sequelae in the reproductive tracts of both males and females, potentially affecting fertility (reviewed in [1,2]). The prevalence of these infections is increasing, and associated reproductive disease poses a huge burden on the economy [3]. While treatment can be initiated and immediately effective, perhaps more than half of all cases are asymptomatic and go undetected (reviewed in [4,5]). Furthermore, early treatment intervention programs can actually increase overall prevalence over time, associated with an increase in susceptibility to secondary infections [6]. Thus, the need for a chlamydial vaccine is undeniable.

A successful vaccine against *Chlamydia trachomatis* infections would need to generate significant antibody and cell-mediated immune responses in the genital mucosa to target both the extracellular Elementary body and the intracellular Reticulate body respectively [7]. Intranasal (IN) immunisation effectively targets antigens to the nasal-associated lymphoid tissues [8], and enhances immunity in the genital tract via the common mucosal immune system [9]. Previous studies have indicated that IN vaccination provides a potent means of targeting protective immunity against sexually transmitted infections to the reproductive tracts of both females and males [10-12]. The benefits of a mucosal vaccination strategy include the potential for self-administration, and the use of non-needle delivery strategies.

A number of adjuvants that are able to induce mucosal immunity are in use in veterinary practice [13], but few have been tested in humans to date. IN immunisation with inactivated influenza vaccine and E.Coli heat-labile toxin (LT) as an adjuvant resulted in patients having a 19-fold greater risk of developing Bell's palsy than unimmunised patients [14]. Another toxin-based adjuvant, cholera toxin (CT) accumulates in the olfactory nerve and bulb of mice [15], as the B subunits of CT and LT bind ganglioside receptors present on all nucleated cells [16]. Thus the use of these agents in human vaccine formulations is currently avoided.

Due to the excellent mucosal adjuvant properties of CT and LT, efforts have been made to develop toxin-based adjuvants with improved safety. CTA1-DD is one such adjuvant, constructed of the A1 subunit of CT directly linked to a dimer of the B cell targeting moiety D, from *Staphylococcus aureus* protein A [17]. The adjuvanticity of CTA1-DD is dependent on the ADP-ribosylating activity of the CT-A1 subunit, and requires direct binding to immunoglobulin on B cells [18,19]. This adjuvant is capable of homing to the mucosal lining near the NALT at a similar time frame to CT [20], and has comparable adjuvanticity [17]. Furthermore, unlike CT or LT, CTA1-DD is safe for use, as it does not accumulate in the olfactory bulbs of mice following IN immunisation [20]. In vitro experiments using human peripheral blood lymphocytes indicate that CTA1-DD is also able to augment the expansion of human B cells as it does in mice [21].

Due to its safety and strong adjuvant properties, CTA1-DD is considered a potential mucosal adjuvant for future use in humans. A number of studies in mice have demonstrated its capacity to induce strong mixed Th1/Th2 immune responses at the lung and gut mucosa when co-administered with antigen and delivered by the IN route. This immunity has resulted in enhanced protection against a number of pathogens including influenza virus, rotavirus and *Helicobacter pylori* [22-24]. However, the capacity of CTA1-DD to enhance mucosal immunity within the genital tract has not yet been examined. Here we demonstrate the first evidence that CTA1-DD immunisation by the IN route is able to induce immunity in the reproductive tract. IN immunisation with chlamydial Major Outer Membrane Protein (MOMP) co-administered with CTA1-DD results in the strong induction of neutralising systemic and mucosal antibodies, and enhances protection against a live challenge with *Chlamydia muridarum,* the murine model for *Chlamydia trachomatis* infection.

Materials and Methods

Mice, antigen, adjuvant and *Chlamydia muridarum*

Female BALB/c mice were obtained from the Animal Resources Centre (Perth, Australia). Mice were provided with sterile food and water *ad libitum*, and were maintained under specific pathogen free conditions. All procedures were approved by the Queensland University of Technology Animal Ethics Committee. The transformed *E.coli* DH5α(pMMM3) expressing the pMAL-c2 vector encoding recombinant maltose binding protein-major outer membrane protein (MOMP) of *Chlamydia muridarum* was a generous gift from Harlan Caldwell (Rocky Mountain Laboratories, Hamilton, Montana) and recombinant protein was prepared as previously described [12,25]. CTA1-DD was prepared as previously described [17] and provided by Nils Lycke. *Chlamydia muridarum (C.mu)* was obtained from the ATCC (VR-123) and was propagated in McCoy cells.

Intranasal Immunization

Groups of five mice were immunized by the intranasal (IN) route with CTA1-DD (20μg) adjuvant alone, 100μg MOMP alone, or CTA1-DD and MOMP in combination. The immunization solution was administered to the external nares in a 5μl volume per nare, under isofluorane anaesthesia. Immunization occurred on days 0, 7 and 14, and animals were boosted on day 35. Serum and lavage samples were collected at sacrifice one week following the final boost according to the methods described in Hickey *et al*.[12]. Briefly, serum was separated from

cardiac puncture blood by centrifugation. Vaginal lavage was obtained by flushing the vagina with 40μl sterile PBS. Uterine lavage was collected by excising the uterine horn at the level of the uterine corpus and removing the ovaries/oviducts, then flushing the horn with 100μl sterile PBS. All samples were stored at -20°C prior to analysis.

Examination of systemic and mucosal MOMP-specific antibodies

MOMP-specific IgG subtype (IgG1 and IgG2a) titres were determined for serum samples using the ELISA methods described in Hickey *et al*.[12]. Vaginal lavage and uterine lavage were analysed for MOMP-specific IgA. Greiner medium-binding 96-well ELISA plates (Greiner Bio-One, FL, USA) were coated with 2μg/well MOMP. Lavage samples were serially diluted two-fold from 1/20 to 1/2560 in PBS-Tween, while serum was diluted 1/100 to 1/800,000 for analysis. The primary antibodies, biotin-conjugated anti-IgG1, anti-IgG2a and anti-IgA, and the secondary Streptavidin-HRP conjugate were all used at a dilution of 1/1000 (Southern Biotechnology Associates, Birmingham AL). The plates were developed using 0.1μg/ml Tetramethyl-benzidine (TMB, Sigma-Aldrich) in phosphate citrate buffer (PCB, Sigma-Aldrich), then stopped by the addition of 1M sulfuric acid. Absorbance of each sample was determined at 450nm and end-point titre (EPT) was defined as the mean dilution at which the absorbance reached that of negative samples (mean PBS sample absorbance + 2 SD).

Neutralization of *Chlamydia muridarum in vitro*

To investigate the *Chlamydia*-neutralizing capacity of these MOMP-specific antibodies, the serum and vaginal lavage samples were then examined by *in vitro* neutralization assay on McCoy cells. Tubes containing 5 x 10^4 inclusion forming units (ifu) of *C.mu* were incubated with two-fold serial dilutions of sample for 1h at 37°C before addition to McCoy cell monolayers in 48-well plates in triplicate (1/10 to 1/1280 for vaginal lavage, 1/20 to 1/2560 for serum). The plates were then incubated for 4h before replacing sample with 500μl Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% FCS and 1μg/ml cycloheximide (Sigma-Aldrich). Infection was then stopped 36h post-inoculation. Chlamydial inclusions were stained using the *Chlamydia* Cel LPS staining kit (CelLabs Brookvale, Australia) and enumerated by microscopy. Percent neutralization was determined by comparison to wells inoculated with *C.mu* alone.

Challenge with *Chlamydia muridarum* and monitoring of clearance

One week following the final boost, groups of five immunized/naïve mice were pretreated with 2.5mg progesterone, then challenged one week later under ketamine/xylazine anaesthesia. Challenge was via direct inoculation of the vaginal vault with 5 x 10^4ifu *C.mu*. Vaginal swabs (Copan nasopharyngeal swabs; Interpath, Australia) were taken every three days up to day 21. Swabs were placed in 500μl SPG solution and stored at -80°C. Mice were sacrificed at day 35 post-infection and gross pathology of the reproductive tract observed. DNA was extracted from swab samples using the Amplicor CT/NG specimen

preparation kit (Roche, Castle Hill, Australia) according to manufacturer's instructions. Real-time PCR to determine chlamydial DNA load in swabs was performed using flanking nucleotide primers specific for *C.mu* MOMP (Forward: 5'-gcc gtt ttg ggt tct gct t-3'; Reverse: 5'-cgt caa tca taa ggc ttg gtt ca-3'), and Platinum Sybr-Green qPCR Supermix with ROX reference dye (Invitrogen, Mount Waverley, Australia). The 25μl reaction volume contained 2μl extracted swab DNA and 20mM each primer, and samples were run and analyzed on ABI PRISM 7000 sequence detection system (PE Applied Biosystems Inc.)[26]. Samples were run in conjunction with a series of standards. Results presented are representative of two separate experiments, and are expressed as number of mice positive for infection. The amounts of shed bacteria between the experiments were not directly comparable, so were unable to be compiled despite showing the same trends.

Statistics

All graphical data are presented as means and SEM. Statistical analyses were performed using GraphPad Prism version 4.0b for Windows (GraphPad Software, San Diego California USA). For ELISA, differences between groups were examined by unpaired two-tailed t-test. *In vitro* neutralization assays and swab clearance were analysed by two-way repeated measures ANOVA with Bonferroni post-test. Significant differences are denoted as *p=0.05 to 0.01, **p=0.01 to 0.001, ***p<0.001.

Results and discussion

MOMP-specific antibodies in serum

IN immunization with CTA1-DD and MOMP resulted in the induction of extremely high levels of circulating IgG antibodies against MOMP (Fig1A). Somewhat unexpectedly, these were no higher than the antibody level induced by IN immunization with MOMP alone, thus it is not possible to determine if this immunization is enhancing Th1 or Th2 responses preferentially. Similar results were found by Akhiani *et al.* [27] who observed only mild increases in specific IgG1 and IgG2a responses when CTA1-DD was added to their IN immunization with *Helicobacter pylori* sonicate [27]. However others have found that the addition of CTA1-DD to antigen can strongly preferentially stimulate an increase in IgG1 denoting a Th2 response [24,28], or an increase in IgG2a denoting a Th1 response [22]. This variable response does not result from the studies being performed in different species of mice, and thus the co-administered antigen must have a role in driving the specific arm of the immune response.

Interestingly despite having similar levels of IgG antibodies, the IN immunization incorporating CTA1-DD was able to induce significantly greater neutralization of chlamydial elementary bodies *in vitro* than immunization with MOMP alone; 68% versus 14% neutralization with a 1/20 dilution of serum sample (Fig1B). The reason for this is uncertain, but could possibly result from CTA1-DD directing immune responses against a different epitope of the MOMP protein, somehow

via their direct interaction with B cells. Another possibility is that antigen recognition occurs in a different manner when CTA1-DD is present. In one study, 20ug CTA1-DD gave significant anti-CTA1 titres in serum and broncho-alveolar lavage, while the adjuvant properties were unaffected [29]. It was proposed that this adjuvant promotes immunity to the co-administered antigen by anti-CTA1 binding to CTA1DD and increasing Fc-receptor-mediated uptake processes [30]. ***I think I need a bit of help with this paragraph please!***

Mucosal reproductive tract antibodies

High levels of MOMP-specific antibodies were induced in the reproductive tracts of mice immunized with CTA1-DD and MOMP, with high titres of IgA detected in the vaginal lavage and uterine lavage (Fig2). High levels of MOMP-specific IgG were also present (data not shown). A one in ten dilution of antibodies in vaginal lavage samples from mice receiving CTA1-DD and MOMP was able to neutralize infectivity of *C.mu* by 34%, while vaginal lavage of control groups was unable to prevent the infection of McCoy cells. This is the first report of CTA1-DD inducing high levels of neutralizing antibodies in the reproductive tract. In fact, in both systemic and mucosal samples, immunization with CTA1-DD and MOMP was able to generate over four times more antibodies than IN immunization with MOMP and a mixture of the adjuvants CT and CpG (data not shown).

This induction of strong neutralizing immunity in the reproductive tract using CTA1-DD as an adjuvant has promising implications for a whole host of

reproductive pathogens. It has previously been shown effective as an adjuvant against respiratory and gastrointestinal viral infections [22-24,31], but this report suggests that CTA1-DD could also be useful for a range of bacterial and viral sexually transmitted infections.

Clearance of *Chlamydia* from the lower reproductive tract

Immunization with MOMP and CTA1-DD resulted in fewer mice infected from day 12 onwards compared to the control groups (Table 1), and a trend for lower levels of shed *Chlamydia*, as shown by McCoy cell culture on swab samples (data not shown). Thus immunizing with CTA1-DD in combination with the antigen has conferred some level of protective immunity, and demonstrated a trend of reduced upper reproductive tract pathology as determined by diameter of oviduct dilation (data not shown). The addition of CTA1-DD in some mouse immunization models has resulted in almost complete protection against oral or respiratory challenge with pathogen [22,24], however, this level of protection is not seen in our model. Immunization studies utilizing MOMP as an antigen show varying levels of protection against an intra-vaginal challenge with *Chlamydia* [32-34]. It is now relatively well accepted that MOMP as a sole antigen will be insufficient to induce complete protective immunity against multiple serovars of *Chlamydia*. Recent studies have indicated the potential use of novel protective antigens including NrdB, CT0512, and CPAF [35-37]. Further studies will need to investigate the protection afforded by such antigens, perhaps as a multi-subunit vaccine formulation, using a suitable delivery route and adjuvant to target

enhanced protective immunity to the genital mucosa, such as the immunization strategy presented in this report.

Recent studies have also demonstrated that direct fusion of antigen/s with CTA1-DD can result in enhanced immunity and protection against challenge [31], while combining antigen with CTA1-DD and ISCOMS enhances immune induction 10-fold [38]. ISCOMS target dendritic cells and possibly macrophages, while CTA1-DD targets antigen to B cells, thus three different types of antigen presenting cells can be utilised [38]. A strong induction of neutralizing antibodies, and reduced numbers of infected mice in response to CTA1-DD and MOMP has been presented in this study, and here we demonstrate for the first time, the compelling potential of this adjuvant for the safe targeting of vaccines against reproductive pathogens.

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Figure legends

Fig 1: Quantitation of neutralizing MOMP-specific IgG in serum. One week post-boost serum samples were collected from five mice per group, and ELISA conducted to determine levels of MOMP-specific IgG1 and IgG2a (A). Absorbance at 450nm was measured and end point titre determined, with t-tests to determine significance. Data presented are representative of two separate experiments. McCoy cell monolayers were inoculated with 5 x 10^4 ifu *C.mu* pre-incubated with serial dilutions of serum to examine *in vitro* neutralization (B). Infection proceeded for 36h before cells were fixed and stained. Percentage neutralization with respect to no serum controls was calculated and significance compared to non-immune serum determined by two-way repeated measures ANOVA with Bonferroni post-test. *p=0.05 to 0.01, **p=0.01 to 0.001, ***p<0.001.

Fig 2: Quantitation of neutralizing MOMP-specific IgA in mucosal samples. Vaginal lavage and uterine lavage samples were collected from five mice per group, and ELISA conducted to determine levels of MOMP-specific IgA (A). Absorbance at 450nm was measured and end point titre determined, with t-tests to determine significance. Data presented are representative of two separate experiments. McCoy cell monolayers were inoculated with 5 x 10^4 ifu *C.mu* pre-incubated with serial dilutions of vaginal lavage to examine *in vitro* neutralization (B). Percentage neutralization with respect to no VL controls was calculated and significance compared to non-immune VL determined by two-way repeated

measures ANOVA with Bonferroni post-test. *p=0.05 to 0.01, **p=0.01 to 0.001, ***p<0.001.

Table 1: Chlamydial clearance determined by PCR on swab samples. Vaginal swabs were collected every three days following intravaginal challenge with *C.mu*. Swab DNA was extracted and chlamydial load determined by Real-Time PCR. Results are representative of two separate experiments, and expressed as number of mice positive for infection.

Figure 1

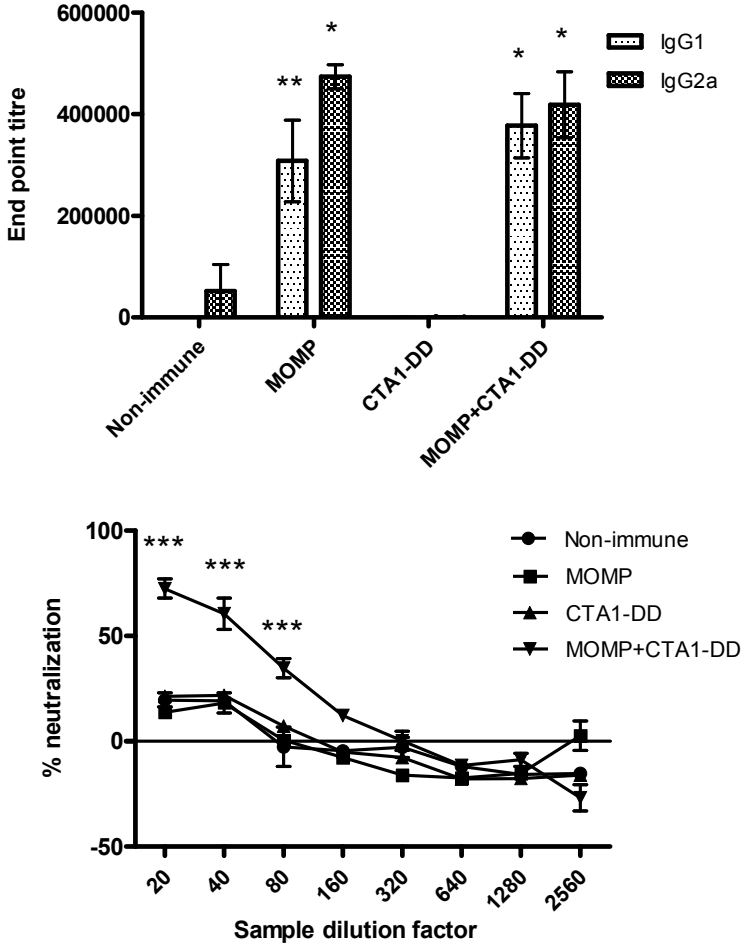
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Figure 2

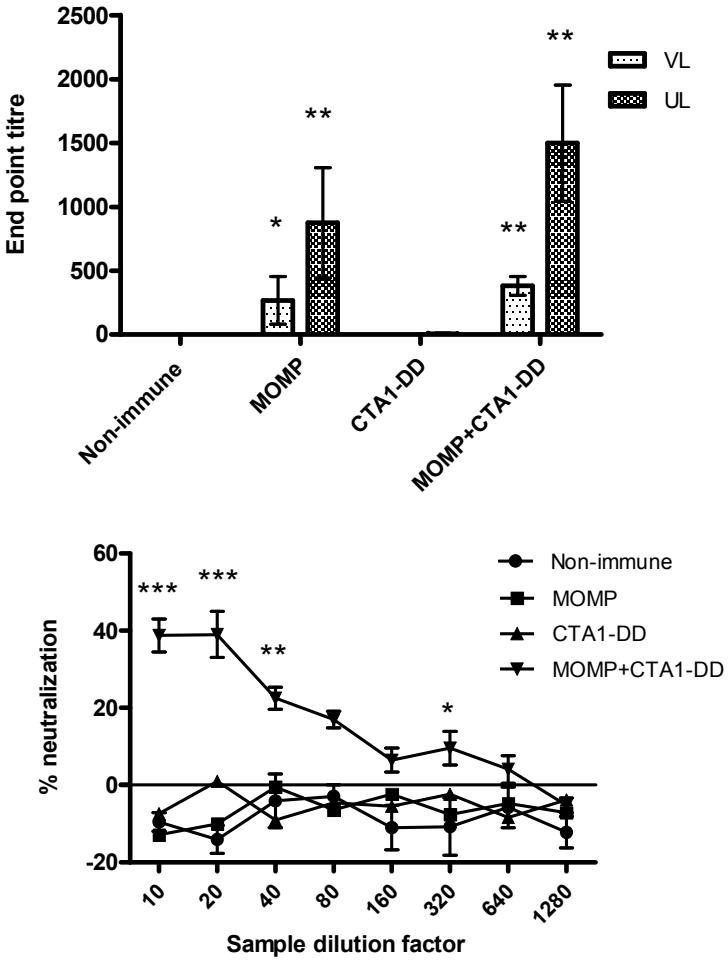
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Table 1

Group	Number of mice positive for infection (>100ifu/swab)						
	3	**6**	**9**	**12**	**15**	**18**	**21**
Non-immune	5	5	5	5	5	3	1
MOMP	5	5	5	4	3	3	0
CTA1DD	5	5	5	5	4	4	0
CTA1DD+MOMP	5	5	5	3	3	1	0