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The Protective Efficacy of Chlamydial Protease-like Activity Factor Vaccination is Dependent upon CD4⁺ T cells

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Abstract

We have previously determined the protective efficacy of intranasal vaccination with chlamydial protease-like activity factor (CPAF) against genital chlamydial infection. Since T-helper 1 (Th1) responses are important for anti-chlamydial immunity, we examined the contribution of CD4⁺ T cells in CPAF mediated immunity against intravaginal (i.vag.) *Chlamydia muridarum* infection in C57BL/6 mice. CPAF+IL-12 vaccination induced antigen-specific CD4⁺ T cells that secreted elevated levels of IFN- γ , and generated strong humoral responses. The protective effects of CPAF vaccination against genital chlamydial challenge were abrogated by anti-CD4 neutralizing antibody treatment. Moreover, anti-chlamydial immunity could be adoptively transferred to naïve recipients using CPAF-specific CD4⁺ T cells. Therefore, CPAF mediated anti-chlamydial immunity is highly dependent upon antigen-specific CD4⁺ T cells.

Keywords

Chlamydia; Vaccine; Chlamydial Protease-like Activity Factor; CD4⁺ T cells; Th1

Introduction

Chlamydia trachomatis is the leading worldwide cause of sexually transmitted bacterial disease [1]. Antimicrobial therapy is available for treatment of genital chlamydial infections; however, the majority of these infections are initially asymptomatic (75% in women and 50% in men) and therefore not recognized [1,2]. Untreated infections may ascend into the upper genital tract and cause chronic inflammatory pathology, leading to sequelae such as pelvic inflammatory disease, ectopic pregnancy and infertility [3]. The rising incidence rates of these infections and sequelae over the last decade [1] underscore the timely importance of development of an effective anti-chlamydial vaccine.

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Anti-chlamydial vaccine candidates have been evaluated for protection in the murine model of genital chlamydial infection [1,2]. Among the few candidates examined, the chlamydial major outer membrane protein (MOMP) has been extensively characterized, but found to be only partially protective [2]. Refolding of MOMP to achieve native conformation prior to immunization has been recently reported to reduce vaginal bacterial shedding and infertility rates in mice after genital *C. muridarum* challenge [4]. Immunization with an anti-idiotypic antibody to the chlamydial exolipid antigen also has been shown to induce partial protection against genital *C. trachomatis* challenge [5]. We recently have demonstrated that intranasal vaccination with the chlamydial protease-like activity factor (CPAF) and the mucosal adjuvant interleukin-12 (IL-12) [6,7] induces enhanced bacterial clearance and robust protection against oviduct pathology following genital *Chlamydia muridarum* challenge (Murthy *et al.*, manuscript in review). The protection induced by CPAF vaccination was shown to be highly dependent on endogenous IFN- γ production. These promising results indicate the importance of additional characterization of CPAF in the induction of protective immunity against genital chlamydial infection.

There is evidence from several laboratories [1,2,9,21,22] to suggest that T helper 1 (Th1) cells acting via IFN- γ -dependent and independent pathways are important for the resolution of genital chlamydial infection. Specifically, depletion of CD4⁺ T cells, but not CD8⁺ T cells or B cells, results in marked inability of mice to resolve primary *C. muridarum* infection in the genital tract [8]. Furthermore, adoptive transfer of *Chlamydia*-specific CD4⁺ T cells, not CD8⁺ T cells or passive transfer of antibodies, confer protective immunity against chlamydial infection [9–12]. Upon bacterial rechallenge, B cell deficient (μ MT) mice depleted of CD4⁺ T cells, but not CD8⁺ T cells, displayed a severe inability to resolve the infection [12], suggesting the importance of CD4⁺ T cells in effective anti-chlamydial immunity. Collectively, these results underscore the importance of antigen-specific CD4⁺ T cells in anti-chlamydial immunity and suggest that vaccination strategies that elicit such responses may be highly beneficial.

In this study, we examined the role of antigen-specific CD4⁺ T cells in CPAF+IL-12 mediated immunity against genital *C. muridarum* infection. Depletion of CD4⁺ T cells significantly abrogated the protective effects of CPAF+IL-12 vaccination. Moreover, protective immunity was shown to be adoptively transferred by CPAF-specific CD4⁺ T cells. These results together suggest that CPAF-mediated immunity is highly dependent on the induction of antigen-specific CD4⁺ T cells.

Materials and Methods

Mice

Female 4–8 week old C57BL/6 mice were purchased from Simonsen Laboratories (Gilroy, CA) and maintained at the University of Texas at San Antonio Animal Facility. Mice were given food and water *ad libitum* and all animal procedures were performed in compliance with the Institutional Animal Care and Use Committee (IACUC) guidelines.

Bacteria

Chlamydia trachomatis mouse pneumonitis (MoPn; recently designated as the separate species *C. muridarum*) was grown on confluent HeLa cell monolayers. The cells were lysed using a sonicator (Fisher, Pittsburgh, PA) and the elementary bodies (EBs) were purified on Renograffin gradients as described previously [13]. Aliquots of bacteria were stored at -70°C in Sucrose-Phosphate-Glutamine (SPG) buffer. *Chlamydia* genus-specific murine monoclonal antibody was used to confirm the identity of the purified bacterium [14].

CPAF

The open reading frames coding for CPAF from *C. trachomatis* L2 genome was cloned into pBAD vectors and expressed as fusion proteins with a 6-His tag at the N-terminus. The amino acid sequences of CPAF share significant identity (82%) between serovar L2 and *C. muridarum* [15]. Expression of the fusion protein designated CPAF (CPAF with 6-His tag) was induced with L-arabinose (Sigma, St. Louis, MO) and the fusion proteins were extracted by lysing the bacteria via sonication in a Triton X-100 lysis buffer (1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 75 U of aprotinin/ml, 20 μ M leupeptin, and 1.6 μ M pepstatin). The fusion protein-containing supernatants were purified further with Ni-NTA agarose beads (QIAGEN, Valencia, CA) for 6-His tag proteins. Aliquots of purified proteins were stored at -70° C until use. Before vaccination, the biological activity of recombinant CPAF was confirmed by the ability to degrade the transcription factor, RFX-5 [16].

Intranasal immunization

Vaccination was performed as described previously [17]. Briefly, mice were anesthetized i.n. with 3% isoflurane using a rodent anesthesia system (Harvard Apparatus, Holliston, MA). Then, the C57BL/6 mice were immunized i.n. on day 0 with 15 μ g CPAF dissolved in 25 μ l sterile phosphate buffered saline (PBS). This was accompanied on days -1 , 0, and $+1$ with 0.5 μ g of recombinant murine IL-12 (Wyeth, Cambridge, MA) in PBS containing 1% normal mouse serum (NMS). Mice were boosted i.n. with 15 μ g CPAF+0.5 μ g IL-12 on days 14 and 28. Some mice received only PBS-NMS (no CPAF vaccine). We have established previously that animals vaccinated with the chosen dose of CPAF and IL-12 exhibited optimal protective immunity against genital chlamydial challenge when compared to treatment with soluble CPAF or IL-12 alone (Murthy *et al.*, manuscript in review). Therefore, this study was restricted to analyses of protective immunity in CPAF+IL-12 vaccinated animals. As previously described, no toxicity was observed with the IL-12 treatment regimen [18].

Antigen-specific CD4⁺ T cell responses

Animals were treated i.n. with CPAF+IL-12 or PBS and spleens were removed after 14 days. Splenocytes were layered over a ficoll density gradient to collect mononuclear cells. CD4⁺ T cell populations were isolated by negative selection using magnetic particles (Stem Cell Technologies) and the purity of CD4⁺ T cell populations was determined to be at least >95% by flow cytometry using an APC labeled anti-CD4 monoclonal antibody (BD Biosciences). A separate pool of naïve splenocytes also was prepared from mock (PBS) vaccinated animals and treated with mitomycin C (25 μ g/ 10^7 cells) for 20 min and used as a source of antigen presenting cells (APCs). Purified CD4⁺ T cells (5×10^5 cells/well) were cultured with naïve antigen presenting cells (5×10^5 cells/well) and stimulated for 72 hr *in vitro* with CPAF or an unrelated antigen, hen egg lysozyme (HEL). Supernatants from the culture wells were analyzed for IFN- γ and IL-4 production using BD OptELISA kits (BD pharmingen, NJ) according to the manufacturer's instructions.

Detection of antibody levels by ELISA

96-well microtiter plates were coated overnight with 5 μ g/ml of CPAF or UV-inactivated *C. muridarum* (10^5 IFU/well) in sodium bicarbonate buffer (pH 9.5), washed with PBS containing 0.05% Tween-20 (Sigma) and blocked for 1 hr at room temperature with PBS containing 2% bovine serum albumin (BSA, EM Science Gibbstown, NJ). Serial dilutions of serum were added to wells and incubated at room temperature for 2 hr. The plates were then washed and incubated for an additional 1 hr with goat anti-mouse total Ig, IgG2a, IgG2b, or IgG1 conjugated to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). After incubation for 1 hr, the plates were washed and *p*-nitrophenyl phosphate substrate was

added for color development. Absorbance at 405 nm was measured using an ELISA microplate reader (Biotek Instruments, Winooski, VT). Reciprocal serum dilutions corresponding to 50% maximal binding were used to obtain titers. No binding of immune sera was observed when the plates were coated with the unrelated antigen HEL.

***In vivo* CD4⁺ T cell depletion**

The GK1.5 hybridoma cell line, which produces anti-CD4 neutralizing antibody [8], was purchased from ATCC and grown according to the manufacturer's instructions. The anti-CD4 monoclonal antibody was purified using ammonium sulfate precipitation. A rat immunoglobulin (rat Ig, Sigma Aldrich) was used as an isotype control. On days -6, -5, and -4 and on the day of challenge and every third day afterwards, animals were injected intraperitoneally (i.p.) with 0.5 mg of purified anti-CD4 monoclonal antibody (GK1.5). The last injection of monoclonal antibody was given on day 21 after challenge. Control mice received rat Ig or PBS injection over the same schedule. CD4⁺ T cell depletion was monitored by flow cytometry using an APC labeled anti-CD4 monoclonal antibody (BD Biosciences). Five days prior to challenge, mice were treated with 2.5 mg of Depo-Provera (Upjohn, Kalamazoo, MI), to synchronize ovulation and animals (now 12–16 wk old) were subsequently infected i.vag. with 1,500 IFU of *C. muridarum*.

Adoptive transfer of CD4⁺ T cells

Experimental mice (4–8 wk old) were immunized intranasally (i.n.) with CPAF+IL-12, while the control groups were challenged i.vag. with 1,500 IFU of *C. muridarum* or mock-treated with PBS. The mice were rested for 30 days after the last boost, sacrificed and the spleens removed. Single cell suspensions were made and layered over a ficoll density gradient (Cedarlane Laboratories, Canada) to obtain mononuclear cells. CD4⁺ T cell populations were enriched by negative selection using magnetic particles (Stem Cell Technologies). The purity of the CD4⁺ T cell population was determined to be at least >95% by flow cytometry using an APC labeled anti-CD4 monoclonal antibody (BD Biosciences). Two hours before transfer, female C57BL/6 mice (4–8 week old) were challenged intra-vaginally with 1,500 IFU of *C. muridarum*. Approximately 10⁷ CD4⁺ T cells were transferred i.p. into naïve mice. Bacterial shedding was monitored at three-day intervals as detailed below.

Intravaginal challenge

Mice were anesthetized i.n. using 3% isoflurane in a rodent anesthesia system (Harvard Apparatus, Holliston, MA) and immediately inoculated i.vag. with 1,500 inclusion forming units (IFU) of *C. muridarum* in 5 µl of sterile SPG buffer. Vaginal vaults of challenged mice were swabbed at three day intervals, and the swabs transferred into Eppendorf tubes containing 4 mm glass beads (Kimble, Vineland, NJ) and 500 µl of sterile SPG buffer. The tubes were vortexed for 1 min and swab material was plated and incubated for 28 hr with HeLa cells grown on coverslips in 24-well plates. The infected HeLa cells were fixed with 2% paraformaldehyde and permeabilized with 2% saponin. Cells were washed using PBS and incubated with Modified Dulbecco's Eagle's Medium containing 10% fetal bovine serum for 1 hr to block non-specific binding. Thereafter, cells were washed and incubated with polyclonal rabbit anti-*Chlamydia* antibody for 1 hr and then incubated for an additional 2 hr with goat anti-rabbit Ig conjugated to FITC (Sigma, St. Louis, MO) plus Hoescht nuclear stain. The treated coverslip cultures were then washed and mounted onto superfrost microscope slides (Fisher) using Fluorsave reagent (Calbiochem, La Jolla, CA). Slides were visualized using a Zeiss Axioskop 2 Plus research microscope (Zeiss, Thornwood, NY). The bacterial shedding was calculated and expressed as the number of inclusion forming units per animal.

Histology and staining

Genital tracts were removed from mice at various time-points after challenge, fixed in 10% neutral formalin, and embedded into paraffin blocks. Serial horizontal sections (5 μ m) were prepared and every tenth section (~8–10 sections per tissue) was stained using hematoxylin and eosin (H&E). Stained sections were visualized using a Zeiss Axioskop 2 Plus research microscope and images were acquired using an Axiocam digital camera (Zeiss, Thornwood, NY). Representative sections stained with H&E were scored in blinded fashion by a trained pathologist using a scoring scheme modified from Rank *et al.* [19]. Dilatation of oviducts was scored as follows: **0**- no significant dilatation, **1**- mild dilatation of single cross-section of oviduct, **2**- 1–3 dilated cross-sections of oviduct, **3**- >3 dilated cross-sections of oviduct, **4**- confluent pronounced dilatation of oviduct. Results are expressed as mean $\pm$ SEM of scores from all animals in a group ($n = 6$).

Statistical analyses

Sigma Stat (Chicago, IL) was used to perform all the tests of significance. The Student *t* test was used to determine differences in cytokine and antibody production, and the Kruskal-Wallis test for differences in vaginal chlamydial shedding between experimental groups. The infection resolution time between groups was compared using the Kaplan-Meier test. Differences were considered statistically significant if *p* values were < 0.05. All data shown are representative of at least two independent experiments.

Results

Intranasal CPAF+IL-12 immunization induces Th1 type immune response

Splenocytes were removed at day 14 after i.n. immunization and purified CD4⁺ T cells (5×10^5 cells/well) were cultured with mitomycin treated splenocytes as antigen presenting cells (5×10^5 cells/well) and stimulated with CPAF. As shown in Fig. 1A, purified CD4⁺ T cells from CPAF+IL-12 vaccinated animals exhibited elevated levels of IFN- γ production, in a dose-dependent fashion (1.2 ng/ml and 1.5 ng/ml of IFN- γ , respectively), upon stimulation with 0.5 μ g or 1 μ g of CPAF, as compared to those from mock-immunized (PBS) animals. Cells cultured with media or the unrelated antigen HEL displayed minimal IFN- γ production. Additionally, there was no induction of cytokine production when purified cells were stimulated with UV-inactivated *C. muridarum* (10^5 IFU/well) or with another 6-Histidine tagged protein (BA1) cloned from *Francisella tularensis* (data not shown), indicating the specificity of measured responses to CPAF. CD4⁺ T cells from all groups of animals responded to the non-specific T cell mitogen concanavalin A (conA) stimulation by producing high levels of IFN- γ (data not shown) indicating that the minimal cytokine production from mock-immunized (PBS) CD4⁺ T cells was not due to an inability of these cells to be activated. There was no detectable IL-4 production in any of the cell cultures (data not shown).

The antibody response to CPAF immunization was measured at timed intervals during the immunization regimen. On day 40, ten days after the last booster immunization, animals vaccinated with CPAF+IL-12 displayed elevated titers of CPAF-specific total Ab and IgG2a, IgG2b and IgG1 antibodies as compared to other treatment groups (Fig. 1B). Additionally, the titers of serum anti-CPAF total Ab (5670 ± 665) and IgG2b (5642 ± 253) were relatively greater than those of IgG2a (3573 ± 916) and IgG1 (3299 ± 1009). There was minimal CPAF-specific antibody response in mock-immunized (PBS) animals and no antibody binding observed in wells coated with HEL (Fig. 1B) or in wells coated with UV-inactivated *C. muridarum* or BA1 (data not shown). Serum antibody levels on days 14 and 28 (data not shown) exhibited comparable trends, but lower titers of each antibody isotype than at day 40. Collectively, these

results demonstrate the induction of antigen-specific Th1 type cellular response, and robust humoral response after CPAF+IL-12 vaccination.

The protective effects of CPAF vaccination is abrogated by depletion of CD4⁺ T cells in vaccinated animals

The contribution of CPAF-specific CD4⁺ T cells in conferring protective immunity against primary genital *C. muridarum* infection was examined by depletion of vaccinated mice using an anti-CD4 neutralizing antibody. Intraperitoneal injection of the neutralizing anti-CD4 antibody markedly depleted the splenic CD4⁺ T cells (<1%) as shown by flow cytometry (Fig. 2A), and in contrast to injection with control rat immunoglobulin (29%). As shown in Fig. 2B, the CPAF+IL-12 vaccinated animals depleted of CD4⁺ T cells shed significantly greater numbers of *Chlamydia* as early as day 9 and through day 24 after challenge, in marked contrast to vaccinated animals injected with control rat immunoglobulin. Specifically, challenged CPAF+IL-12 animals treated with control rat Ig shed significantly fewer chlamydiae on day 9 (~10-fold reduction), day 12 (~50 fold reduction), and day 15 (~10,000 fold reduction), as compared to challenged CPAF+IL-12 animals treated with anti-CD4 antibody (Fig. 2B). Whereas all of the CPAF+IL-12 vaccinated animals treated with control rat immunoglobulin completely resolved the infection by day 15 after challenge, 100% of similarly vaccinated mice treated with anti-CD4 antibody were still shedding *Chlamydia* as late as day 24 after challenge (Fig. 2C). Vaccinated CD4⁺ T cell depleted mice resolved the infection only upon cessation of the neutralizing anti-CD4 treatment (Fig. 2B & 2C). Mock-immunized animals shed significantly fewer *Chlamydia* on days 18–24 as compared to CPAF+IL-12 vaccinated anti-CD4 antibody treated mice (Fig. 2B) and completely resolved the infection by day 27 after challenge. These results suggest that CPAF+IL-12 mediated chlamydial clearance is dependent on CD4⁺ T cells.

Anti-chlamydial immunity can be adoptively transferred by CPAF-specific CD4⁺ T cells

Based upon the outcome of the depletion studies, we examined whether adoptive transfer of CPAF-specific CD4⁺ T cells would confer protection against genital chlamydial challenge. Naïve recipient C57BL/6 mice were injected i.p. with enriched CD4⁺ T cells obtained from mice vaccinated with CPAF+IL-12 and concurrently challenged i.vag. with 1,500 IFU of *C. muridarum*. As controls, some groups of mice received enriched CD4⁺ T cells purified from mice infected i.n. with *C. muridarum* (200 IFU) or treated with PBS (mock) and concurrently challenged with *Chlamydia*. As shown in Fig. 3A and 3B, *C. muridarum* challenged mice which received CPAF-specific CD4⁺ T cells exhibited significantly lower bacterial shedding at the indicated time-points compared to recipients of T cells from mock-immunized (PBS) animals, with 50% of the CPAF-specific CD4⁺ T cell recipient animals completely resolving the infection by day 15 and 100% by day 18. In comparison, mock-immunized (PBS) CD4⁺ T cell recipients exhibited greater shedding through 24 days and resolved the infection only by day 27–30 after challenge (Fig. 3B). Importantly, *C. muridarum* challenged mice that received CPAF-specific CD4⁺ T cells exhibited resolution kinetics comparable to mice that received *C. muridarum*-specific CD4⁺ T cells (Fig. 3A & 3B), with both groups of animals completely resolving the infection by day 18 post-challenge. These results demonstrate that (a) anti-chlamydial protective immunity can be adoptively transferred by CPAF-specific CD4⁺ T cells and (b) CPAF-specific CD4⁺ T cells induce protective anti-chlamydial immunity comparable to *C. muridarum*-specific CD4⁺ T cells.

CPAF-specific CD4⁺ T cells are capable of inducing protective immunity against oviduct pathology

The development of oviduct dilatation is a characteristic complication of genital chlamydial infection in mice [2]. We previously have found that intranasal CPAF+IL-12 vaccination protects against development of oviduct dilatation and reduces inflammatory cellular infiltration into the upper genital tract following genital *C. muridarum* challenge in an IFN- γ dependent fashion (Murthy *et al.*, manuscript in review). In the current study, we examined the contribution of CPAF-specific CD4⁺ T cells in protection against oviduct pathology by analyzing the development of histopathological changes after *C. muridarum* challenge in animals that were vaccinated and depleted of CD4⁺ T cells or in mice that received CPAF-specific CD4⁺ T cells. As shown in Fig. 4B, CPAF+IL-12 vaccinated mice treated with control rat Ig exhibited significantly lower oviduct dilatation scores when compared to mock-immunized (PBS) animals at day 80 after challenge. In marked contrast, CPAF+IL-12 vaccinated mice treated with anti-CD4 antibody exhibited greater oviduct pathology than mock-immunized (PBS) animals. Additionally, *C. muridarum* challenged mice adoptively transferred with CPAF-specific CD4⁺ T cells exhibited significantly lower oviduct pathology than mock-immunized (PBS) animals. The degree of protection against the development of oviduct dilatation in CPAF+IL-12 (rat Ig group) and in animals receiving CPAF-specific CD4⁺ T cells was comparable to animals receiving *C. muridarum* primed T cells. Moreover, CPAF+IL-12 vaccinated mice (rat Ig group) and those adoptively transferred with CPAF-specific or *C. muridarum*-specific CD4⁺ T cells exhibited significantly lower infiltration of polymorphonuclear leukocytes, mononuclear cells, and plasma cells on day 80 after challenge, when compared to mock-immunized animals (data not shown). These results together suggest that CD4⁺ T cells play an important role in CPAF+IL-12 vaccination-induced protection against oviduct pathology.

Discussion

We previously have shown that CPAF+IL-12 vaccinated animals display faster resolution, but not resistance, to genital chlamydial infection suggesting that cellular immunity, not neutralizing antibodies, may be involved in mediating this protective effect. In the current study, we examined the role of CD4⁺ T cells in CPAF+IL-12 mediated immunity against genital chlamydial infection. CPAF+IL-12 vaccination induced the generation of IFN- γ producing antigen-specific CD4⁺ T cells and resulted in enhanced resolution of genital chlamydial infection in C57BL/6 mice. Depletion of CD4⁺ T cells from such immunized animals abrogated the protective effects of vaccination, whereas adoptive transfer of CPAF-specific CD4⁺ T cells induced enhanced resolution of genital *C. muridarum* infection.

Splenocytes from CPAF+IL-12, but not mock (PBS) vaccinated animals displayed high levels of IFN- γ and minimal IL-4 production upon *in vitro* stimulation with CPAF, demonstrating the induction of an Ag-specific Th1 type cellular response. There is accumulated evidence to suggest that Th1 responses and IFN- γ production are important for optimal resolution of genital chlamydial infection [1,2]. To this end, *Chlamydia*-specific Th1 clones, but not Th2 clones, have been demonstrated to be capable of adoptively transferring anti-chlamydial immunity [9–11]. Conversely, MHC class II deficient, but not MHC class I deficient animals, displayed an inability to resolve a primary chlamydial infection [20]. Mice with a targeted disruption in IFN- γ production (IFN- γ ^{-/-} mice) have also been shown to display a marked inability to resolve chlamydial infection and prevent bacterial dissemination [21]. In this regard, the bactericidal effect of IFN- γ on intracellular *Chlamydia* in epithelial cell cultures has been shown to occur via the indoleamine-2,3-dioxygenase pathway in human cells versus p47 GTPases in murine

cells [22]. Thus, induction of Th1 responses is an essential component of an effective anti-chlamydial vaccine.

CPAF+IL-12 vaccinated animals exhibited high titers of serum anti-CPAF total Ab and IgG2b, and lower titers of IgG2a and IgG1 at day 40 after immunization. However, the contribution of these antibodies to protective immunity has yet to be characterized. Other studies suggest a possible role for antibodies in immunity against chlamydial infections. B cell deficient (μ MT) animals, but not wild type animals displayed a severe inability to resolve chlamydial reinfection in the absence of CD4⁺ T cells, suggesting an important role for B cells in anti-chlamydial immunity [8,23]. Additionally, mice deficient in Fc receptors have been shown to exhibit reduced antigen-specific T cell responses and sub-optimal resolution of infection following rechallenge [24,25]. Specifically, Fc receptors of IgG2a (Fc γ RII) and IgA (Fc α) antibodies has been shown to be important for efficient chlamydial antigen presentation to T cells.

The enhanced resolution of genital chlamydial infection and protection against oviduct dilatation induced by CPAF+IL-12 vaccination was abrogated by depletion of CD4⁺ T cells. In addition, protective immunity could be adoptively transferred by CPAF-specific CD4⁺ T cells. Taken together with the fact that CPAF+IL-12 mediated immunity is highly dependent on endogenous IFN- γ production, these results suggest that CPAF induced anti-chlamydial immunity may be highly dependent on IFN- γ secreting Th1 type CD4⁺ T cells. The findings that CPAF-specific CD4⁺ T cells are important for induction of protective immunity has bearing on several aspects of chlamydial vaccine development for humans including (a) route of vaccination and type of adjuvant(s) required to elicit the appropriate type of immune response at the site of infection and (b) identification of MHC class II restricted protective epitopes within CPAF for development of subunit vaccines. To this end, we have recently found that CpG deoxynucleotides are also highly effective Th1 adjuvants when used with CPAF for induction of protective immunity against genital chlamydial infection in mice (Cong and Arulanandam, unpublished observations). Furthermore, we have determined that CPAF contains human HLA-DR4 (MHC class II) restricted epitopes that are capable of generating protective immunity against chlamydial infection (Murthy *et al.*, In Press, Infection and Immunity).

Adoptive transfer of CPAF-specific CD4⁺ T cells induced comparable protective immunity against genital challenge to that induced by transfer of CD4⁺ T cells primed by *C. muridarum* infection suggesting that CPAF may be a dominant chlamydial antigen for induction of protective immune responses. To this end, *Chlamydia* sero-positive humans have been shown to exhibit higher titers of serum antibodies against CPAF than the highly expressed surface exposed antigens, such as MOMP and Hsp60 [26]. Importantly, recombinant CPAF cloned from *C. trachomatis* L2 induced protective immunity against *C. muridarum* challenge. CPAF derived from these two strains display approximately 82% amino acid identity [15] with greater homology between human serovars suggesting that the protective CPAF epitopes may be conserved across the species and serovars of *Chlamydia*. In addition, monoclonal antibodies raised against serovar L2 CPAF have been shown to recognize CPAF from either serovar L2 or *C. muridarum* [15], indicating a high degree of immunological cross-reactivity. In contrast, the most extensively characterized chlamydial vaccine candidate MOMP is homotypic and does not afford cross-protection [2]. Additionally, MOMP has been shown to be predominantly a B cell immunogen [1,2] and needs refolding before vaccination to induce protective immunity [4].

In summary, we have described a defined chlamydial vaccine candidate (CPAF) that induces CD4⁺ T cell dependent protective immunity. The possibility of using CPAF as an alternative

to, or in combination with, MOMP to induce robust anti-chlamydial immunity is currently being explored.

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Fig 1. Intranasal CPAF+IL-12 vaccination induces robust Th1 cellular and humoral response
(A) Cytokine recall responses after immunization. Animals (5 mice/group) were treated i.n. with CPAF + IL-12 or PBS. On day 14, spleens were collected and enriched CD4⁺ T cell populations were tested for CPAF-induced IFN- γ production by ELISA. **(B)** Systemic anti-CPAF Ab responses after immunization. Animals (10 mice/group) were bled on day 40 after primary immunization. Serum anti-CPAF antibody isotype levels were analyzed by ELISA using CPAF-coated microtiter plates. Results are expressed as means $\pm$ SEM of reciprocal serum dilutions corresponding to 50% maximal binding. Results are representative of two independent experiments. * Significant differences between IFN- γ production by CD4⁺ T cells from animals vaccinated with CPAF+IL-12 group and those treated with PBS (mock) ($P < 0.05$, Student t -test).

Fig 2. CPAF induced protection is dependent on CD4⁺ T cells

Animals (6 mice/group) were treated with three doses of CPAF+IL-12 or PBS. One month following the final vaccination, mice were challenged i.vag. with 1,500 IFU of *C. muridarum* and vaccinated animals were treated either with rat anti-mouse neutralizing anti-CD4 Ab or control rat Ig on 6, 5, and 4 days before challenge, on the day of challenge, and subsequently on the indicated days. At every third day following challenge, chlamydial shedding was measured. The splenic CD4⁺ T cell population, after neutralizing anti-CD4 Ab or control rat Ig treatment, was determined by flow cytometry at every time-point following treatment but (A) shown as representative populations after treatment. (B) Numbers of chlamydial inclusion forming units (IFUs) recovered from vaginal swabs at the indicated days after genital challenge. Results are expressed as means $\pm$ SEM of the bacterial recovery from all animals in a group. * Significant differences between challenged CPAF+IL-12/rat Ig treated group and other experimental groups ($P < 0.05$, Kruskal-Wallis test). * Significant differences between challenged CPAF+IL-12/anti-CD4 Ab treated group and other experimental groups ($P < 0.05$, Kruskal-Wallis test). (C) Percentage of animals shedding *Chlamydia* after genital challenge. Significant differences were detected in the time required for resolution of infection between challenged CPAF+IL-12/rat Ig treated versus CPAF+IL-12/anti-CD4 Ab treated animals. ($P < 0.001$, Kaplan-Meier test). Results are representative of two independent experiments.

Fig 3. Anti-chlamydial immunity can be adoptively transferred by CPAF-specific CD4⁺ T cells Naïve recipient animals (6 mice/group) were injected i.p. with enriched CD4⁺ T cells (10⁷ cells/animal) collected from mice 40 days after treatment with either CPAF+IL-12, PBS, or i.n. infection with *C. muridarum* (200 IFU/animal). Concurrently, animals were challenged i.vag. with 1,500 IFU of *C. muridarum*. At the indicated days following challenge, chlamydial shedding was measured. (A) Numbers of chlamydial inclusion forming units (IFUs) recovered from vaginal swabs at the indicated days after genital challenge. Results are expressed as means $\pm$ SEM of the bacterial recovery from all animals in a group. * Significant differences between challenged PBS CD4⁺ T cell recipient and other experimental groups ($P < 0.05$, Kruskal-Wallis test). (B) Percentage of animals shedding *Chlamydia* after genital challenge. Significant differences were detected in time required for resolution of infection between challenged PBS CD4⁺ T cell recipient mice and other experimental groups ($P < 0.001$, Kaplan-Meier test). Results are representative of two independent experiments.

Fig 4. CPAF-induced protection against genital pathology is dependent on CD4⁺ T cells

Animals (6 mice/group) were treated with three doses of CPAF+IL-12 or PBS. One month following the final vaccination, mice were challenged i.vag. with 1,500 IFU of *C. muridarum* and CPAF+IL-12 immunized animals were treated either with rat anti-mouse neutralizing anti-CD4 Ab or control rat Ig. Conversely, naïve recipient animals (6 mice/group) were injected with enriched CD4⁺ T cells (10^7 cells/animal) collected from animals 40 days after i.vag. infection with *C. muridarum* (1,500 IFU/animal) or treatment with CPAF+IL-12. The development of oviduct dilatation was scored on day 80 after challenge by microscopic examination and results expressed as means $\pm$ SEM of the oviduct dilatation scores. * Significant differences between indicated groups and challenged PBS treated group ($P < 0.05$, Kruskal-Wallis test). Results are representative of two independent experiments.