Published in final edited form as:

Vaccine. 2013 November 19; 31(48): . doi:10.1016/j.vaccine.2013.09.036.

A T Cell Epitope-Based Vaccine Protects Against Chlamydial Infection in HLA-DR4 Transgenic Mice

Weidang Li^a, Ashlesh K. Murthy^b, Gopala Krishna Lanka^a, Senthilnath L Chetty^a, Jieh-Juen Yu^a, James P. Chambers^a, Guangming Zhong^c, Thomas G. Forsthuber^a, M. Neal Guentzel^a, and Bernard P. Arulanandam^{a,*}

^aSouth Texas Center for Emerging Infectious Diseases and Center for Excellence in Infection Genomics, Department of Biology, University of Texas at San Antonio, San Antonio, TX 78249.

^bDepartment of Pathology and Department of Dental Medicine, Midwestern University, Downers Grove, IL, 60515.

^cDepartment of Microbiology and Immunology, University of Texas Health Science Center, San Antonio, TX 78229.

Abstract

Vaccination with recombinant chlamydial protease-like activity factor (rCPAF) has been shown to provide robust protection against genital *Chlamydia* infection. Adoptive transfer of IFN-competent CPAF-specific CD4⁺ T cells was sufficient to induce early resolution of chlamydial infection and reduction of subsequent pathology in recipient IFN- γ -deficient mice indicating the importance of IFN- γ -secreting CD4⁺ T cells in host defense against *Chlamydia*. In this study, we identify CD4⁺ T cell reactive CPAF epitopes and characterize the activation of epitope-specific CD4⁺ T cells following antigen immunization or *Chlamydia* challenge. Using the HLA-DR4 (HLA-DRB1*0401) transgenic mouse for screening overlapping peptides that induced T cell IFN- γ production, we identified at least 5 CPAF T cell epitopes presented by the HLA-DR4 complex. Immunization of HLA-DR4 transgenic mice with a rCPAFep fusion protein containing these 5 epitopes induced a robust cell-mediated immune response and significantly accelerated the resolution of genital and pulmonary *Chlamydia* infection. rCPAFep vaccination induced CPAF-specific CD4⁺ T cells in the spleen were detected using HLA-DR4/CPAF-epitope tetramers. Additionally, CPAF-specific CD4⁺ clones could be detected in the mouse spleen following *C. muridarum* and a human *C. trachomatis* strain challenge using these novel tetramers. These results provide the first direct evidence that a novel CPAF epitope vaccine can provide protection and that HLA-DR4/CPAF-epitope tetramers can detect CPAF epitope-specific CD4⁺ T cells in HLA-DR4 mice following *C. muridarum* or *C. trachomatis* infection. Such tetramers could be a useful tool for monitoring CD4⁺ T cells in immunity to *Chlamydia* infection and in developing epitope-based human vaccines using the murine model.

© 2013 Elsevier Ltd. All rights reserved.

*Address correspondence and reprint requests to Dr. Bernard Arulanandam, Department of Biology, University of Texas at San Antonio, One UTSA circle, San Antonio, TX 78249. Bernard.arulanandam@utsa.edu; Ph: (210)458-5492; Fax: (210)458-5523.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of interest: The authors have no potential conflicts of interest to report.

Authors' contribution

WL, AKM, JPC, GZ, TGF, MNG and BPA conceived and designed the study; WL, GKL, SLC and JPC performed the study; WL, JY, MNG, and BPA wrote the paper. All authors have read and approved the final manuscript.

1. Introduction

Chlamydia trachomatis causes the most common bacterial sexually transmitted disease worldwide [1, 2]. Despite the many efforts that have been made over several decades to develop a protective *C. trachomatis* vaccine, there currently is no efficacious vaccine available. In the early vaccine trials, use of whole chlamydial agents as *Chlamydia* vaccines proved to be of limited value since protection was short lived and some vaccines produced exaggerated ocular pathology over the long term. [3, 4]. This prompted the search for the formulation of a subunit vaccine, which would elicit a specific immune response to unique protein(s) and provide protection without stimulating pathological immunity. Several potential vaccine candidates (MOMP, CPAF, PMP, PorB) eliciting reactive CD4⁺ T cells and/or antibody responses have been evaluated in a mouse model [5–9] and all showed partial protection against genital *Chlamydia* infection. Thus, the identification of specific-protective epitopes that could be used either as an alternative equivalent to those already characterized proteins, or in combination with multi-epitopes from multi-antigens, becomes a sound strategy for developing an effective *Chlamydia* vaccine.

Chlamydia muridarum, originally isolated from the lungs of mice but sharing a very high similarity in gene order and content with the human pathogen *C. trachomatis* serovar D (average only ~10% difference in orthologous genes [10]), was used to establish the mouse genital chlamydial infection model [11] which mimics many aspects of acute genital infection with *C. trachomatis* in women [12]. Using this murine model, we have demonstrated that the recombinant chlamydial protease-like activity factor (rCPAF) is an effective *Chlamydia* vaccine antigen. Specifically, we showed that vaccination with rCPAF derived from the human infectious *C. trachomatis* L2 serovar (approximately 82% identity and 90% similarity to *C. muridarum* ortholog), provided protection (acceleration of bacterial clearance, reduction of upper genital tract pathology, and preservation of fertility) against intravaginal (i.vag.) *C. muridarum* challenge in conventional C57BL/6 mice and the HLA-DR4 transgenic mice (HLA-DR4tg), suggesting that CPAF may also induce a protective immune response in humans [7, 13–19]. We further demonstrated that rCPAF mediated protection was dependent upon CD4⁺ T cells and required IFN- [6, 20].

Here, we report the mapping of CPAF T cell epitopes and the application of the identified epitopes in the development of a novel fusion-peptide vaccine and in the detection of CPAF-specific T cell clones, following chlamydial infection or CPAF vaccination, using MHC class II tetramers. By identifying CPAF epitopes and tracking CD4⁺ T cells during *Chlamydia* infection, we may be able to provide new insights into protective mechanisms and enhance the development of a potential effective T cell epitope vaccine against *Chlamydia* associated diseases.

2. Materials and methods

2.1. Bacteria and cells

Chlamydia muridarum Nigg was grown on confluent HeLa cell monolayers. Elementary bodies (EBs) were purified by discontinuous density gradient centrifugation as described previously [15]. EBV-transfected HLA-DR4 (DRB1*0401) homozygous B cells were used for HLA-DR4 molecular purification [21].

2.2. Mice, immunizations, and infections

HLA-DRA-IE /HLA-DRB1*0401-IE transgenic (HLA-DR4tg) mice [13, 22] were bred at the University of Texas at San Antonio. Animal care and experimental procedures were performed in compliance with the Institutional Animal Care and Use Committee (IACUC) guidelines. Female transgenic mice (6–10 week-old) were immunized with protein antigens,

including *C. trachomatis* L2 rCPAF [13], by subcutaneous (s.c.) or intranasal (i.n.) routes as described previously [14]. Intranasal infection with live *C. muridarum* EBs induces nearly-complete protective immunity against subsequent genital or respiratory challenge, and therefore was used as a positive control for vaccine efficacy in these experiments. All mice are anesthetized for i.n. administration. *Chlamydia muridarum* pulmonary and vaginal infection was achieved by the i.n. [23] or intravaginal (i.vag.) [7] challenge route with 5×10^2 and 5×10^4 IFU of bacteria, respectively, in 5 μ l of SPG buffer. *Chlamydia trachomatis* infection was achieved by primary intravaginal challenge using *C. trachomatis* serovar D (1×10^6 IFU in 5 μ l of SPG buffer) on day 0, followed by a secondary intravaginal CT-D challenge at 3 weeks following the primary challenge, and a tertiary challenge at 2 weeks following the secondary challenge. In order to render the mice anestrous and more receptive to the genital infection, animals were injected s.c. with 2.5 mg of Depoprovera (medroxy-progesterone acetate; Pharmacia) on day 5 before challenge. Vaginal chlamydial shedding and pulmonary *Chlamydia* burdens were measured as described in our previous reports [13, 23].

2.3. CPAF mapping

Overlapping CPAF peptides (20 mer) were synthesized by New England Peptide for determinant mapping according to the *C. trachomatis* L2 CPAF sequence. The IFN-ELISPOT assay was performed as described previously [24]. Briefly, 96-well MultiScreen-HTS filtration plates (Millipore) were coated overnight at 4 °C with 2 μ g/ml murine IFN-specific mAb (eBioscience, clone AN-18). Splenocytes isolated from 3 rCPAF vaccinated, or 4 *C. muridarum* infected mice, in HL-1 medium (Fisher) were added separately from individual animals to the coated plates at 5×10^5 cells per well in the presence of individual peptides at 10 μ g/ml or *Chlamydia* at 5×10^5 IFU per well. After 20 h of incubation at 37 °C with 5% CO₂, the plates were washed and then incubated with biotinylated IFN-specific mAb (eBioscience, clone R4-6A2) at 0.5 μ g/ml. This was followed by incubation with streptavidin-alkaline phosphatase (Dako) at a 1/1000 dilution. The spots were visualized with BCIP/NBT substrate (KPL) and enumerated with an ImmunoSpot Series 3 Analyzer (Cellular Technology).

2.4. rCPAFep cloning and purification

To express a novel CPAF epitopes protein (rCPAFep), we constructed an expression vector which contained 2 copies of 5 CPAF epitope genes (Cep1-5) in tandem as illustrated in Fig. 2A. Briefly, 997 nucleotides were synthesized (GenScript) to contain sequences encoding each of the 5 selected CPAF epitopes flanked by an N-terminal li-Key (LRMKLPKS) for enhancing epitope presentation and a C-terminal spacer (GPGPG) to link each epitope, as well as a His (6 $\times$) tag at the C-terminus of the rCPAFep for easy protein purification [25]. The synthetic CPAFep gene was cloned to a pET23a vector and used to transform an *E. coli* Rosetta strain (Novagen) for protein expression. rCPAFep expression was induced in the presence of 1 μ M IPTG, with purification under denaturing conditions using an Ni-NTA Agarose column (Qiagen) according to the manufacturer's guidelines.

2.5. Antigen-specific CD4⁺ T cell responses

HLA-DR4tg mouse spleens (n=3) were removed at day 14 post i.n. vaccination with rCPAFep plus 10 μ g CpG containing oligodeoxynucleotide (CpG) or CpG alone (mock), and single splenocyte cell suspensions were prepared. CD4⁺ T cells were enriched using a CD4 Enrichment Kit (STEMCELL) and the purity was determined to be at least 90% by flow cytometry. A pool of naïve monocytes treated with radiation (3000 rads) was used as a source of APCs. The purified CD4⁺ T cells (5×10^5 cells/well) were cultured with APCs (1×10^5 cells/well) and stimulated for 72 h *in vitro* with rCPAF (2 μ g/ml), rCPAFep (2 μ g/ml), or the unrelated antigen hen egg lysozyme (HEL, 2 μ g/ml), in 96 well culture plates.

Supernatants from the triplicate culture wells were analyzed for IFN- γ production using a BD OptELISA kit (BD Pharmingen).

2.6. HLA-DR4 tetramer staining and flow cytometry analysis

HLA-DR4 (DRB1-0401) tetramers containing PE fluorochrome labeled CPAF peptides (Cep 2 and Cep3; sequences described in supplementary Table 1) or control peptide (CLIP) were generated by the NIH Tetramer Core Facility. Cep2 tetramer and Cep3 tetramer were synthesized individually and were mixed together only to better stimulate/bind CD4⁺ T cells. Spleens were removed from HLA-DR4tg mice on day 21 post subcutaneous rCPAFep (100 μ g) plus CFA (1 mg/ml emulsion) vaccination, or on day 12 after *Chlamydia* challenge (i.vag, 5 $\times$ 10⁴ IFU). Single splenocyte cell suspensions were prepared. CD4⁺ T-cells were enriched and stimulated with rCPAFep protein or peptides in the presence of irradiated autologous APCs (antigen presenting cells) in complete RPMI 1640 medium containing rhIL-2 (5 ng/ml) and rhIL-7 (1 ng/ml) for 8 days for T cell expansion before tetramer staining. Antigen stimulated CD4⁺ T cells were incubated in complete RPMI 1640 medium for 3 h with tetramers (4 μ g/ ml) at 37 °C, unless otherwise indicated, and subjected to flow cytometry analysis (ISRII, BD Biosciences) to identify the CPAF-specific CD4⁺ T cell population with FITC-conjugated anti-CD4 Ab (Bio-legend).

2.7. Statistical analyses

Sigma Stat (Systat Software Inc.) was used to perform all tests of significance. Student's t test was used for comparisons between two groups and analysis of variance (ANOVA) between multiple groups for vaginal chlamydial shedding. Differences between groups were considered statistically significant if *p* values were <0.05. All data shown are representative of 2–3 independent experiments and each experiment shown was analyzed independently.

3. Results

3.1. Identification of putative MHC class II-restricted CPAF epitopes using HLA-DR4tg mice

Chlamydia CPAF protein was expressed in mice during chlamydial genital tract infection, as seen in the columnar cells of the endocervix and endometrium (Supplementary Fig.1), and has been shown to stimulate robust host immune responses [13, 26]. To identify CPAF CD4⁺ T cell epitopes restricted by human HLA-DR4 molecules, HLA-DR4tg mice were immunized s.c. with the full length rCPAF protein derived from *C. trachomatis* plus CFA adjuvant. Twenty-one days after the injection, splenocytes were used for *in vitro* sensitization with synthetic overlapping peptides derived from CPAF. In order to select highly reactive T cell epitopes, we screened for IFN- γ production in ELISPOT assays. This selection was based on an initial screening of peptides that stimulated greater than 25 IFN-spots per 5 $\times$ 10⁵ splenocytes (Fig. 1, upper panel). Among the 9 peptides identified, 8 were overlapping reactive fragments thereby representing only 4 non-overlapping peptides (Cep1-4) and Cep5 was a single strong reactive peptide without adjacent reactive overlapping peptides. Collectively, we selected a total of 5 peptides (aa sequences are listed in Supplementary Table 1) from initial screening for further evaluation of immunogenicity and protective immunity. Minimal IFN- γ secreting spots were detected upon stimulation with unrelated peptides, in contrast to high numbers of IFN- γ spots in response to stimulation with overlapping CPAF peptides, indicating that CPAF epitopes presented by HLA-DR4 molecule were specifically recognized by T cells. Splenocytes from mock (CFA) vaccinated HLA-DR4 mice produced a baseline of IFN- γ spots following stimulation by synthetic overlapping CPAF peptides (data not shown). Although *C. muridarum* Cep1-5 aa sequences are slightly different from the *C. trachomatis* derived rCPAF (Supplementary Table 1), these 5 CPAF peptides also can stimulate the T cells obtained from the *C.*

muridarum infected mice to produce IFN- γ , suggesting that Cep1-5 are processed by APCs and presented to T cells during primary *C. muridarum* infection (Fig. 1, lower panel).

3.2. Resolution of genital *C. muridarum* challenge in HLA-DR4 tg mice after vaccination with rCPAFep

We expressed a novel CPAF epitope fusion protein (rCPAFep) consisting of 2 copies of each Cep1-5 and a C-terminus His (6 $\times$)-tag as illustrated in Figure 2A. Then a 34 kDa protein was over-expressed in transformed *E. coli* upon IPTG induction, purified to high homogeneity by Nickel affinity chromatography and shown to have positive reactivity with anti-His-tag antibody (Figure 2B).

We then assessed the immunogenicity of rCPAFep and its protective efficacy against genital chlamydial challenge. First, HLA-DR4tg mice were vaccinated i.n. with rCPAFep plus CpG, or CpG alone, and CD4 $^{+}$ T-cells were enriched from splenocytes at day 14 for cellular cytokine recall assay. As shown in Figure 2C, rCPAFep primed splenocytes secreted high levels of IFN- γ when stimulated with either the full length rCPAF (518 $\pm$ 148 pg/ml) or the combined epitope protein rCPAFep (1225.3 $\pm$ 394.2 pg/ml), in contrast to a negligible reaction with HEL suggesting that vaccination with rCPAFep may induce robust antigen-specific Th1 type mediated immune responses required for *Chlamydia* clearance. Next, we compared the protective efficacy mediated by rCPAF and rCPAFep vaccination. As shown in Figure 3, CpG (mock)-vaccinated mice displayed high levels of vaginal chlamydial shedding initially and progressively cleared the infection with complete clearance by day 30 after challenge. Consistent with our previous observations [15], live EB vaccinated mice started to clear the infection as early as day 3 with marked reduction of chlamydial shedding at day 6, and complete clearance in all mice by day 9. rCPAF+CpG vaccinated mice displayed significant reductions in chlamydial shedding, with clearance of the infection in 83% of the mice as early as day 18, and in all mice by day 21 after challenge. Similarly, 50% of rCPAFep+CpG vaccinated mice cleared the infection at day 18 with all mice free of vaginal bacterial shedding by day 24.

3.3. Acceleration of Chlamydia clearance in the lungs by rCPAFep vaccination

A second chlamydial infection model was used to evaluate the protective efficacy of rCPAFep against *C. muridarum*. We observed that *Chlamydia* replicated in the lungs during the first week of pulmonary infection for all vaccinated (s.c. rCPAF, s.c. rCPAFep, i.n. Live EB) groups and PBS mock (s.c.) treated mice (Fig. 4). While the bacterial numbers in mock treated mice continued to increase at day 10, a significant decrease of bacterial burdens was evident in both rCPAF and rCPAFep vaccinated mice and almost no viable *Chlamydia* could be detected in EB vaccinated mice at day 10. By day 14, all vaccinated mice cleared the infection while mock treated mice still sustained a high bacterial load in the lungs. The comparable protective efficacy of the peptide-fusion rCPAFep and full length rCPAF against pulmonary chlamydial infection was consistent with their comparable protection against genital infection.

3.4. Direct detection of specific CD4 $^{+}$ T cells after rCPAFep vaccination or *C. muridarum* infection

One of the applications of identified T cell epitopes is to generate MHC tetramers for identifying/tracking antigen-specific T cells. To serve this purpose, we chose Cep2 and 3 (Cep2/3) in this study for further characterization since they are part of the mature protein (Cep1 was within the predicted signal peptide), and stimulated T cell activation better than Cep4 and 5 (Fig. 1, lower panel) with chlamydial infection. Cep2 and Cep3 were shown to tightly bind in the peptide-binding groove of HLA-DR4 with a K_d of 0.30 μ M and 0.85 μ M, respectively (Supplementary Fig. 2). Cep2, Cep3/HLA-DR4 tetramers were generated to

detect CPAF-specific CD4⁺ T cells. Since antigen-specific T cell frequency is very low, we combined Cep2 and 3 tetramers (both PE-labeled and mixed at a 1:1 ratio) in this study to maximize the detection capability. As shown in Figure 5A, direct staining of purified CD4⁺ T cells, following rCPAFep+CFA s.c. vaccination or *C. muridarum* vaginal infection, with Cep2/3 tetramers showed 3.6% of CD4⁺ T cells bound with Cep2 and Cep3 HLA-DR4 tetramers from a representative rCPAFep vaccinated mouse and 2.3% of CD4⁺ T cells from a *C. muridarum* challenged mouse. The frequency of CD4⁺ T cells binding in either the mock (CFA) vaccinated or mock (PBS) challenged mice was significantly lower (0.5% and 0.3%, respectively). The calculated frequency of Cep2/3 positive CD4⁺ T-cells from all analyzed mice (3–4 per group) are summarized in the Figure 5A bar graph, indicating the presence of $3.5 \pm 0.1\%$ and $1.9 \pm 0.6\%$ CPAF-specific T cells in the rCPAFep vaccinated and *C. muridarum* infected mice, respectively. These results demonstrated that the novel Cep tetramers are useful reagents to identify and enumerate the CPAF-specific CD4⁺ T cells induced by the rCPAFep vaccine or by *Chlamydia* infection.

To further confirm that the CD4⁺ T cells stained by the Cep2/3-HLA-DR4 tetramers represented CPAF epitope-specific CD4⁺ T cell populations, we expanded the CD4⁺ T cells *in vitro* by specific-epitope stimulation. As shown in Figure 5B, Cep2/3-HLA-DR4 tetramers bound to 25% and 17% of CD4⁺ T cells from a representative rCPAFep vaccinated and a *C. muridarum* challenged mouse, respectively. Overall, the frequency of detected tetramer positive CD4⁺ T cells (Fig 5B, 3–4 mice per group) after *in vitro* stimulation of total splenic CD4⁺ T cells with rCPAFep demonstrated a dramatic increase in mice previously vaccinated with rCPAFep or infected with *C. muridarum*, but increased minimally in CFA (adjuvant only) or PBS (mock) immunized mice. This suggests that despite a low frequency of Cep2/3 positive CD4⁺ T cells *in vivo* following vaccination or infection, they can be expanded significantly by *in vitro* peptide stimulation demonstrating the antigen-specificity of the response.

3.5. *C. trachomatis* intra-vaginal infection leads to CPAF CD4⁺ T cell activation and proliferation

Since we successfully demonstrated that the Cep tetramers can be used to detect CPAF specific CD4⁺ T cells, we examined whether chlamydial CPAF was expressed in the HLA-DR4tg mice, processed and presented by APCs, and stimulated CPAF specific CD4⁺ T cell activation and proliferation during genital infection with the human trachomatis serovar D. The splenocytes were isolated, stimulated and stained with Cep2/3 tetramers on day 12 after the third *C. trachomatis* challenge. As shown in Figure 6A, following direct staining of T cells from *C. trachomatis* infected mice with Cep2/3 tetramers, only 0.9% CD4⁺ T cells bound the Cep2 and Cep3-HLA-DR4 tetramers from one of the *C. trachomatis* infected mice, but this increased to 6.5% after expansion *in vitro*. Almost no CD4⁺ T cells bound to the unrelated control tetramers. Overall, significantly higher numbers of CPAF-specific CD4⁺ T cells were detected in *C. trachomatis* infected mice, compared to PBS mock challenged mice (Fig. 6B). Collectively, we have provided evidence that CPAF specific CD4⁺ T cells were activated and may be expanded during human *Chlamydia* strain infection in the HLA-DR4tg mice, which strongly supports the feasibility of developing a T cell epitope-based vaccine against *Chlamydia* infection.

4. Discussion

In previous studies, our laboratory has demonstrated that vaccination of HLA-DR4tg mice with rCPAF can significantly accelerate the resolution of genital *C. muridarum* infection, whereas similarly vaccinated MHC class II deficient mice failed to resolve the infection [13]. These results indicated the presence of protective HLA-DR4 epitopes within the CPAF protein, and provided a viable platform for mapping those CPAF CD4⁺ T cell epitopes. We

now report the identification of 5 CPAF derived T cell reactive epitopes (Cep1-5) which, when expressed as a fusion protein (rCPAFep), provide a comparable protective efficacy against pulmonary and genital chlamydial infection to full length CPAF, suggesting that these 5 peptides are the dominant protective epitopes of the CPAF protein.

One of the advantages of administering a rCPAFep epitope-based vaccine over full length rCPAF is that the former, with reduction of non-necessary protein sequences, may induce less undesirable effects while retaining the capability to induce comparable protective immunity against *Chlamydia* infection. In addition, this vaccine also could be combined with other potent chlamydial antigens for formulating a better composite vaccine candidate. For example, the combination of rCPAFep with B-cell epitopes from MOMP, which has been shown to induce neutralizing antibody [27], may provide a higher degree of protective efficacy against a primary chlamydial infection and limit immunopathological consequences.

The chlamydial HLA-DR4tg mouse infection model is an excellent surrogate experimental animal system to develop a vaccine against human *Chlamydia* diseases. In this model, all MHC class II-presented responses are induced via human HLA-DR4 molecules. rCPAFep vaccinated HLA-DR4tg mice exhibited significantly accelerated resolution of the genital infection and CD4⁺ T cells also exhibited the highest antigen-specific IFN- γ production upon stimulation with rCPAFep, compared to mock immunized mice, indicating that protective immunity was induced primarily via the HLA-DR4 molecules. Thus, this model overcomes the often doubted human relevance of testing chlamydial vaccine candidates for protection via endogenous mouse MHC class II molecules.

HLA-DR4/epitope tetramers are useful reagents for direct visualization, characterization and isolation of *Chlamydia*-specific CD4⁺ T cells, which may serve as an immune correlate for effective vaccine induced protection. Our present data provided the first direct evidence that CPAF epitope-specific CD4⁺ T cells were induced in the spleen after either vaccination or *Chlamydia* infection. Although the percentage of tetramer stained positive splenic CD4⁺ T cells was low, rCPAFep vaccination still provided protection against *C. muridarum* infection. In this regard, a recent report showed that only 1000 *Chlamydia*-specific T cells constitute the lower limit needed for significant protection in mice; although, those mice were infected by a newly developed transcervical (intrauterine) inoculation method [28]. Thus, the small population of *Chlamydia*-specific CD4⁺ T cells induced by rCPAFep vaccination may be sufficient to confer protective immunity following *Chlamydia* challenge.

Several studies have shown that i.vag. challenge with *C. trachomatis* in mice induced less immune cell infiltration and did not produce strong protective immunity [29–31]. However, adaptive immune responses could be mounted after repeated *C. trachomatis* challenge in mice. Thus, we were able to detect 0.9% of CD4⁺ T cells bound to Cep2 and Cep3/HLA-DR4 tetramers in HLA-DR4tg mice after 3 *C. trachomatis* serovar D challenges. This is the first evidence that a conserved CPAF epitope is presented by the host following both *C. muridarum* and *C. trachomatis* infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the NIH Tetramer Core Facility for providing tetramers Cep2 and Cep3 for this study. This work was supported by National Institutes of Health Grant 1RO1AI074860 and the Army Research Office of the Department

of Defense under Contract No. W911NF-11-1-0136. This project also was supported by a grant from the National Institute on Minority Health and Health Disparities (G12MD007591) from the National Institutes of Health.

Abbreviations used in this paper

CPAF	chlamydial protease-like activity factor
rCPAFep	recombinant CPAF-epitope fusion protein
IFU	inclusion-forming unit
HLA-DR4tg mice	HLA-DR4 transgenic mice

References

1. Satterwhite CL, Gottlieb SL, Romaguera R, Bolan G, Burstein G, Schuler C, et al. CDC Grand Rounds: Chlamydia Prevention: Challenges and Strategies for Reducing Disease Burden and Sequelae (Reprinted from MMWR, vol 60, pg 370–373, 2011). *Jama-J Am Med Assoc.* 2011; 305(17):1757–1759.
2. Morrison RP, Caldwell HD. Immunity to murine chlamydial genital infection. *Infect Immun.* 2002; 70(6):2741–2751. [PubMed: 12010958]
3. Dhir SP, Agarwal LP, Detels R, Wang SP, Grayston JT. Field trial of two bivalent trachoma vaccines in children of Punjab Indian villages. *Am J Ophthalmol.* 1967; 63 Suppl(5):1639–1644. [PubMed: 4960885]
4. Wilsmore AJ, Wilsmore BC, Dagnall GJ, Izzard KA, Woodland RM, Dawson M, et al. Clinical and immunological responses of ewes following vaccination with an experimental formalin-inactivated *Chlamydia psittaci* (ovis) vaccine and subsequent challenge with the live organism during pregnancy. *Br Vet J.* 1990; 146(4):341–348. [PubMed: 2397374]
5. Pal S, Theodor I, Peterson EM, de la Maza LM. Immunization with an acellular vaccine consisting of the outer membrane complex of *Chlamydia trachomatis* induces protection against a genital challenge. *Infect Immun.* 1997; 65(8):3361–3369. [PubMed: 9234798]
6. Li W, Murthy AK, Guentzel MN, Seshu J, Forsthuber TG, Zhong G, et al. Antigen-specific CD4+ T cells produce sufficient IFN-gamma to mediate robust protective immunity against genital *Chlamydia muridarum* infection. *J Immunol.* 2008; 180(5):3375–3382. [PubMed: 18292563]
7. Murthy AK, Chambers JP, Meier PA, Zhong G, Arulanandam BP. Intranasal vaccination with a secreted chlamydial protein enhances resolution of genital *Chlamydia muridarum* infection, protects against oviduct pathology, and is highly dependent upon endogenous gamma interferon production. *Infect Immun.* 2007; 75(2):666–676. [PubMed: 17118987]
8. Ifere GO, He Q, Igietseme JU, Ananaba GA, Lyn D, Lubitz W, et al. Immunogenicity and protection against genital *Chlamydia* infection and its complications by a multisubunit candidate vaccine. *J Microbiol Immunol Infect.* 2007; 40(3):188–200. [PubMed: 17639158]
9. Yu H, Jiang X, Shen C, Karunakaran KP, Jiang J, Rosin NL, et al. *Chlamydia muridarum* T-cell antigens formulated with the adjuvant DDA/TDB induce immunity against infection that correlates with a high frequency of gamma interferon (IFN-gamma)/tumor necrosis factor alpha and IFN-gamma/interleukin-17 double-positive CD4+ T cells. *Infect Immun.* 2010; 78(5):2272–2282. [PubMed: 20231405]
10. Read TD, Brunham RC, Shen C, Gill SR, Heidelberg JF, White O, et al. Genome sequences of *Chlamydia trachomatis* MoPn and *Chlamydia pneumoniae* AR39. *Nucleic Acids Res.* 2000; 28(6): 1397–1406. [PubMed: 10684935]
11. Barron AL, White HJ, Rank RG, Soloff BL, Moses EB. A new animal model for the study of *Chlamydia trachomatis* genital infections: infection of mice with the agent of mouse pneumonitis. *J Infect Dis.* 1981; 143(1):63–66. [PubMed: 7217713]
12. Morrison RP, Feilzer K, Tumas DB. Gene knockout mice establish a primary protective role for major histocompatibility complex class II-restricted responses in *Chlamydia trachomatis* genital tract infection. *Infect Immun.* 1995; 63(12):4661–4668. [PubMed: 7591120]

13. Murthy AK, Cong Y, Murphey C, Guentzel MN, Forsthuber TG, Zhong G, et al. Chlamydial protease-like activity factor induces protective immunity against genital chlamydial infection in transgenic mice that express the human HLA-DR4 allele. *Infect Immun*. 2006; 74(12):6722–6729. [PubMed: 17015458]
14. Cong Y, Jupelli M, Guentzel MN, Zhong G, Murthy AK, Arulanandam BP. Intranasal immunization with chlamydial protease-like activity factor and CpG deoxynucleotides enhances protective immunity against genital *Chlamydia muridarum* infection. *Vaccine*. 2007; 25(19):3773–3780. [PubMed: 17349723]
15. Li W, Guentzel MN, Seshu J, Zhong G, Murthy AK, Arulanandam BP. Induction of cross-serovar protection against genital chlamydial infection by a targeted multisubunit vaccination approach. *Clin Vaccine Immunol*. 2007; 14(12):1537–1544. [PubMed: 17942608]
16. Chaganty BK, Murthy AK, Evani SJ, Li W, Guentzel MN, Chambers JP, et al. Heat denatured enzymatically inactive recombinant chlamydial protease-like activity factor induces robust protective immunity against genital chlamydial challenge. *Vaccine*. 2010; 28(11):2323–2329. [PubMed: 20056182]
17. Li W, Murthy AK, Guentzel MN, Chambers JP, Forsthuber TG, Seshu J, et al. Immunization with a combination of integral chlamydial antigens and a defined secreted protein induces robust immunity against genital chlamydial challenge. *Infect Immun*. 2010; 78(9):3942–3949. [PubMed: 20605976]
18. Murthy AK, Li W, Guentzel MN, Zhong G, Arulanandam BP. Vaccination with the defined chlamydial secreted protein CPAF induces robust protection against female infertility following repeated genital chlamydial challenge. *Vaccine*. 2011; 29(14):2519–2522. [PubMed: 21300093]
19. Li W, Murthy AK, Chaganty BK, Guentzel MN, Seshu J, Chambers JP, et al. Immunization with dendritic cells pulsed ex vivo with recombinant chlamydial protease-like activity factor induces protective immunity against genital *Chlamydia muridarum* challenge. *Front Immunol*. 2011; 2:73. [PubMed: 22566862]
20. Murphey C, Murthy AK, Meier PA, Guentzel MN, Zhong G, Arulanandam BP. The protective efficacy of chlamydial protease-like activity factor vaccination is dependent upon CD4+ T cells. *Cell Immunol*. 2006; 242(2):110–117. [PubMed: 17116296]
21. Forsthuber TG, Shive CL, Wienhold W, de Graaf K, Spack EG, Sublett R, et al. T cell epitopes of human myelin oligodendrocyte glycoprotein identified in HLA-DR4 (DRB1*0401) transgenic mice are encephalitogenic and are presented by human B cells. *J Immunol*. 2001; 167(12):7119–7125. [PubMed: 11739534]
22. Murthy AK, Chaganty BK, Li W, Guentzel MN, Chambers JP, Seshu J, et al. A limited role for antibody in protective immunity induced by rCPAF and CpG vaccination against primary genital *Chlamydia muridarum* challenge. *FEMS Immunol Med Microbiol*. 2009; 55(2):271–279. [PubMed: 19281569]
23. Murthy AK, Sharma J, Coalson JJ, Zhong G, Arulanandam BP. *Chlamydia trachomatis* pulmonary infection induces greater inflammatory pathology in immunoglobulin A deficient mice. *Cell Immunol*. 2004; 230(1):56–64. [PubMed: 15541719]
24. Forsthuber T, Yip HC, Lehmann PV. Induction of TH1 and TH2 immunity in neonatal mice. *Science*. 1996; 271(5256):1728–1730. [PubMed: 8596934]
25. Hurtgen BJ, Hung CY, Ostroff GR, Levitz SM, Cole GT. Construction and evaluation of a novel recombinant T cell epitope-based vaccine against *Coccidioidomycosis*. *Infect Immun*. 2012; 80(11):3960–3974. [PubMed: 22949556]
26. Sharma J, Bosnic AM, Piper JM, Zhong G. Human antibody responses to a *Chlamydia*-secreted protease factor. *Infect Immun*. 2004; 72(12):7164–7171. [PubMed: 15557641]
27. Cunningham KA, Carey AJ, Lycke N, Timms P, Beagley KW. CTA1-DD is an effective adjuvant for targeting anti-chlamydial immunity to the murine genital mucosa. *J Reprod Immunol*. 2009; 81(1):34–38. [PubMed: 19501917]
28. Gondek DC, Olive AJ, Stary G, Starnbach MN. CD4+ T cells are necessary and sufficient to confer protection against *Chlamydia trachomatis* infection in the murine upper genital tract. *J Immunol*. 2012; 189(5):2441–2449. [PubMed: 22855710]

29. Morrison SG, Farris CM, Sturdevant GL, Whitmire WM, Morrison RP. Murine *Chlamydia trachomatis* genital infection is unaltered by depletion of CD4⁺ T cells and diminished adaptive immunity. *J Infect Dis.* 2011; 203(8):1120–1128. [PubMed: 21321103]
30. Lyons JM, Igietsme JU, Black CM, Morre SA. Identification of candidate genes using the murine model of female genital tract infection with *Chlamydia trachomatis*. *Drugs Today (Barc).* 2009; 45(Suppl B):51–59. [PubMed: 20011695]
31. Ramsey KH, Cotter TW, Salyer RD, Miranpuri GS, Yanez MA, Poulsen CE, et al. Prior genital tract infection with a murine or human biovar of *Chlamydia trachomatis* protects mice against heterotypic challenge infection. *Infect Immun.* 1999; 67(6):3019–3025. [PubMed: 10338514]

Highlight

HLA-DR4 restricted T cell epitopes of CPAF were mapped

CPAF epitope fusion protein protects against genital and pulmonary chlamydial challenge

CPAF-specific CD4⁺ T cells were identified using HLA-DR tetramers

Figure 1.

Mapping of HLA-DR4 (DRB1*0401)-restricted CPAF epitopes. Six- to 10-wk old HLA-DR4tg mice were immunized s.c. with rCPAF plus CFA (n=3) or challenged i.vag. with *C. muridarum* (n=4). Single cell suspensions prepared from individual spleens (21 days after treatment) were stimulated with CPAF overlapping peptides (20-mer), unrelated peptides or positive control stimuli (CD3 and rCPAF) and subjected to ELISPOT assay to measure the frequencies of epitope-specific IFN- γ producing T cells. * indicates 5 selected CPAF T cell reactive epitope peptides for the construction of rCPAFep, a novel CPAF epitope fusion protein. # indicates 2 peptides chosen for the generation of HLA-DR4 tetramers.

Figure 2.

Vaccination of HLA-DR4tg mice with rCPAFep induced T cell immune responses. (A) Illustration of the rCPAFep construct, which consists of 2 tandem repeats of 5 CPAF epitopes (Cep1-5; not all the duplicated peptides sequences are shown), their flanking Li-key and spacer, and a His (6×) tag. (B) rCPAFep (arrows) was over-expressed upon IPTG induction in the transformed *E. coli* host (left panel, commassie blue stained SDS-PAGE gel), purified to high homogeneity (middle, commassie blue stained SDS-PAGE gel), and reacted with anti-His antibody (right, Western blot). (C) Pooled purified CD4⁺ T cells from rCPAFep + CpG i.n. vaccinated, or CpG mock treated HLA-DR4tg, mouse (n=3 per group, day 14) were incubated with irradiated naïve monocytes (as APCs) and stimulated by rCPAF, rCPAFep, or HEL (an unrelated control antigen) for 72 h. IFN-γ in culture supernatants was measured by ELISA and presented as mean ± SD. * Significant ($p < 0.05$; T test) difference between the indicated groups.

Figure 3.

Vaccination of HLADR4tg mice with rCPAFep protects against genital chlamydial infection comparable to full-length CPAF immunization. Groups (6 per group) of HLA-DR4tg mice were immunized i.n. with rCPAF/CpG, rCPAFep/CpG, or CpG (mock), and boosted twice at a two week intervals. Another group (n = 6) of mice received one live EB i.n. vaccination. One month after the final immunization, mice were challenged i.vag. with 5×10^4 IFU of *C. muridarum*. Chlamydial shedding was monitored every third day after challenge for 1 month, and presented as mean $\pm$ SD for each group at each time point (A). * Significant reductions ($P < 0.05$; one-way ANOVA) in bacterial shedding between the indicated group and CpG-immunized (mock) mice are indicated. (B). Percentage of mice shedding *Chlamydia* after genital challenge for each vaccination group is summarized.

Figure 4.

Vaccination with rCPAFep protects against pulmonary chlamydial infection comparable to full-length CPAF immunization. Groups (15 per group) of mice were immunized s.c. with rCPAF, rCPAFep, or PBS (mock), and boosted twice at a two week intervals. Another group (n = 15) of mice received one live EB i.n. vaccination. One month after the final immunization, mice were challenged i.n. with 5×10^2 IFU of *C. muridarum*. Chlamydial burdens in the lungs were measured at the indicated times and presented as mean $\pm$ SD for each group at each time point. * Significant reductions ($P < 0.05$; one-way ANOVA) in bacterial shedding between the indicated group and mock-immunized (mock) mice are indicated.

Figure 5.

Detection of CPAF specific CD4⁺ T cell populations induced by rCPAFep vaccination or *C. muridarum* infection using tetramer staining. (A) Representative flow cytometry quadrant plots showing frequency of Cep2/3 HLA-DR4 tetramer stained CD4⁺ T cells in the right top quadrant for a mock (CFA adjuvant alone) and rCPAFep vaccinated mouse (upper panels), or a mock (PBS) and *C. muridarum* infected mouse (lower panels). The frequency (mean±SD) of Cep2/3-specific CD4⁺ cells was calculated from 3–4 individually tested mice of each indicated group (bar graph). (B) Representative flow cytometry quadrant plots showing frequency of Cep2/3 HLA-DR4 tetramer stained CD4⁺ T cells upon *in vitro* expansion by rCPAFep stimulation or peptide. The frequency (mean±SD) of Cep2/3-specific CD4⁺ cells

in expanded cell cultures was calculated from 3–4 individually tested mice of each indicated group. * Significant ($p < 0.05$; T test) difference between the indicated groups.

Figure 6.

Detection of CPAF specific CD4⁺ T cell populations induced by *C. trachomatis* infection. (A) Representative flow cytometry quadrant plots showing frequency of Cep2/3 HLA-DR4 tetramer stained CD4⁺ T cells in the right top quadrant for a mock (PBS) and *C. trachomatis* infected mouse (n=3 per group; tested individually) without (upper panels) or with (lower panels) *in vitro* expansion by rCPAFep stimulation or peptides. (B) The frequency (mean±SD) of Cep2/3-specific CD4⁺ cells was calculated from 3 individually tested mice of each indicated group. * Significant ($p < 0.05$; T test) difference between the indicated groups.