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Intranasal immunization with recombinant chlamydial protease-like activity factor attenuates atherosclerotic pathology following *Chlamydia pneumoniae* infection in mice

Weidang Li^{#1}, Pareesha Gudipaty^{#2}, Chuxi Li^{3,4}, Kyle K. Henderson³, Kyle H. Ramsey^{1,3}, and Ashlesh K. Murthy^{1,†}

¹College of Veterinary Medicine, Midwestern University, Glendale 85308

²College of Health Sciences, Midwestern University, Glendale 85308

³Chicago College of Osteopathic Medicine, Midwestern University, Downers Grove, 60515, USA

⁴College of Pharmacy, The University of Texas at Austin, 78712, USA

These authors contributed equally to this work.

Abstract

We have shown previously that intranasal vaccination with recombinant chlamydial protease-like activity factor (rCPAF: antigen) and interleukin-12 (IL-12) as an adjuvant induces robust protection against pathological consequences of female genital tract infection with *Chlamydia muridarum*, a closely related species and a rodent model for the human pathogen *Chlamydia trachomatis*. Another related species *Chlamydia pneumoniae*, a human respiratory pathogen, has been associated with exacerbation of atherosclerotic pathology. CPAF is highly conserved among *Chlamydia spp.* leading us to hypothesize that immunization with rCPAF with IL-12 will protect against high-fat diet (HFD) and *C. pneumoniae*-induced acceleration of atherosclerosis. rCPAF ±IL-12 immunization induced robust splenic antigen (Ag)-specific IFN- γ and TNF- α production and significantly elevated serum total anti-CPAF Ab, IgG2c, and IgG1 antibody levels compared to mock or IL-12 alone groups. Addition of IL-12 to rCPAF significantly elevated splenic Ag-specific IFN- γ production and IgG2c/IgG1 anti-CPAF antibody ratio. Following intranasal *C. pneumoniae* challenge and HFD feeding, rCPAF±IL-12-immunized mice displayed significantly enhanced splenic IFN- γ , not TNF- α , response on days 6 and 9 after challenge, and significantly reduced lung chlamydial burden on day 9 post-challenge compared to mock- or IL-12-immunized mice. Importantly, rCPAF±IL-12-immunized mice displayed significantly reduced atherosclerotic pathology in the aortas after *C. pneumoniae* challenge. Serum cholesterol levels were comparable between the groups suggesting that the observed differences in pathology were due to protective immunity against the infection. Together, these results confirm and extend our previous observations that CPAF is a promising candidate antigen for a multi-subunit vaccine regimen to protect against *Chlamydia*-induced pathologies, including atherosclerosis.

[†]Corresponding Author: Ashlesh K. Murthy, M.D., Ph.D., Department of Pathology and Population Medicine, College of Veterinary Medicine, Midwestern University, 19555, N 59th Ave, Glendale, AZ 85308., Ph: (623) 572 3299, Fax: (623) 537 6399, amurth@midwestern.edu.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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We have now demonstrated that rCPAF with or without IL-12 vaccination significantly attenuates *C. pneumoniae*-induced exacerbation of atherosclerosis in the C57BL/6 high-fat diet-fed male mouse model. The results of this study confirm our previous demonstrations of the protective efficacy of rCPAF against disease sequelae induced by *C. muridarum*, and extend those reports by demonstrating efficacy against a human pathogen *C. pneumoniae*, and against disease sequelae in the cardiovascular system. These results further provide compelling evidence for inclusion of CPAF into a multi-subunit vaccine against a wide range of Chlamydia-induced disease sequelae in humans.

Keywords

Chlamydia pneumoniae; atherosclerosis; Chlamydial protease-like activity factor; protective immunity

INTRODUCTION

The genus *Chlamydia* contain various species that are obligate intracellular pathogens of humans. *Chlamydia trachomatis* sexually transmitted infections can lead to severe chronic pathologies including infertility in the female upper reproductive tract (reviewed in ¹). A preventive vaccine that is thought to be the ideal solution to this problem has yet to be developed. We have provided compelling evidence previously that intranasal (i.n.) immunization with recombinant chlamydial protease-like activity factor (rCPAF) cloned from *C. trachomatis* L2 genome, with murine recombinant interleukin-12 (IL-12) or CpG deoxynucleotides (CpG-ODN) as adjuvant, significantly enhances clearance of chlamydial infection and reduces upper reproductive tract pathologies in the mouse as well as the guinea pig models of genital chlamydial infection ²⁻⁴.

C. pneumoniae mostly causes upper respiratory tract infections but is also a cause of community acquired pneumonia worldwide ^{5, 6}. Forty to seventy percent of population display anti-*C. pneumoniae* antibody and patients with ischemic heart disease, prior myocardial infarction and atherosclerosis are more likely to be sero-positive for *C. pneumoniae* ⁷⁻⁹. Interestingly, the organism can be detected in fatty streaks and atherosclerotic plaques, not in normal vessels ¹⁰⁻¹². Although three large antibiotic treatment trials failed to reduce the risk of secondary coronary events ^{13, 14}, a lead investigator of at least one of those trials, and others, have been careful to point out that those studies have been misinterpreted that the ineffectiveness of antibiotic treatment disproved any causality and that the studies as designed did not exclude a role for *C. pneumoniae* in the initiation,

progression, or exacerbation of atherosclerosis¹⁵. Thus, an association between *C. pneumoniae* and exacerbation of atherosclerosis has clearly been documented based on multiple lines of evidence in human studies whereas a direct cause-and-effect relationship has yet to be conclusively determined. Nevertheless, in rabbits or mice fed a hyperlipidemic diet, exacerbation of atherosclerosis can clearly be induced following intravascular or intranasal *C. pneumoniae* infection, respectively^{16, 17}, thereby fulfilling many of Koch's original postulates and their revisions by Fredricks and Relman¹⁸.

As CPAF is a highly conserved protein among the genus *Chlamydia*¹⁹, we hypothesized that immunization with rCPAF+IL-12 would also induce protective immunity against *C. pneumoniae* infection and atherosclerotic pathology.

RESULTS

Humoral response following immunization.

Mice were immunized i.n. with PBS (mock), IL-12 alone, rCPAF alone, or rCPAF+IL-12, on day 0, 14, and 28, respectively. The serum anti-CPAF antibody responses were measured on day 40. As shown in Figure 1, animals vaccinated with rCPAF±IL-12 displayed significantly elevated titers of serum anti-rCPAF total antibody, IgG1, and IgG2C compared to mock- or IL-12 alone-immunized groups, which exhibited minimal anti-CPAF antibodies. Furthermore, rCPAF+IL-12 immunized mice displayed significantly elevated titers of anti-CPAF IgG2c and Ig2c-IgG1 ratio when compared to rCPAF alone-immunized animals, suggesting that the adjuvant skews toward a T-helper 1 type immune response. These data indicate that i.n. immunization with *C. pneumoniae* rCPAF+IL-12 induces strong humoral responses against rCPAF.

Cellular cytokine responses following immunization.

C57BL/6 mice were immunized i.n. with PBS (mock vaccinated), IL-12, rCPAF, and rCPAF+IL-12. Fourteen days later, splenocytes were stimulated *in vitro* with rCPAF and antigen (Ag) specific cytokine production was measured by ELISA. As shown in Figure 2, splenocytes from rCPAF±IL-12-vaccinated mice exhibited significantly greater IFN- γ and TNF- α production compared to those from mock (PBS)- or IL-12-immunized animals. Furthermore, rCPAF+IL-12 immunization induced significantly greater Ag-specific IFN- γ , not TNF- α , production compared to immunization with rCPAF alone. These results demonstrate the induction of a robust Ag-specific cellular IFN- γ response, which has been shown to be required for protective immunity against *C. pneumoniae*²⁰, following i.n. rCPAF+IL-12 immunization.

Lung chlamydial burden challenged animals.

Mice were immunized i.n. with PBS (mock), IL-12, rCPAF, and rCPAF+IL-12. One month following the final vaccination, mice were challenged with *C. pneumoniae*. On days 3, 6, 9, and 12 after intranasal challenge, *C. pneumoniae* burden in the lungs were measured. As shown in Figure 3a, rCPAF+IL-12-vaccinated mice displayed significantly reduced lung bacterial burden on day 9 compared to mock- or IL-12-immunized animals. As expected based on our previous findings in the genital challenge model³, lung bacterial burden was

comparable in all groups of animals on days 3 and 6 following challenge. Infection was below detectable levels in all groups on day 12 after challenge.

Cellular cytokine responses following chlamydial challenge.

We evaluated Ag-specific cytokine production from splenocytes on days 3, 6, and 9 after bacterial challenge. The cytokine response on day 3 after challenge was below detection range of our assay in all groups of animals. *In vitro* stimulation with rCPAF induced minimal IFN- γ production on day 6 (Figure 3b), and increased levels were detected on day 9 (Figure 3c) after challenge, in mock- or IL-12-immunized animals. In comparison, rCPAF $\pm$ IL-12 immunized animals displayed significantly greater IFN- γ production following *in vitro* stimulation with rCPAF, on days 6 and 9 after challenge, when compared to mock- or IL-12-immunized animals. On the contrary, minimal Ag-specific cytokine response was observed from splenocytes of each group when stimulated with UV-inactivated *Chlamydia pneumoniae* elementary bodies (UV-EB; data not shown). This was expected because CPAF is expressed only in the reticulate body phase and secreted into host cytosol, not in the EB phase. Furthermore, Ag-specific TNF- α levels tended to be higher in rCPAF $\pm$ IL-12-immunized animals when compared to mock- or IL-12-immunized animals on days 6 and 9 after challenge, the differences were not statistically significant. These results suggest that enhanced clearance of bacteria from the lungs observed on day 9 after challenge correlates with cellular IFN- γ response against CPAF, not against the integral chlamydial antigens in UV-EB.

Atherosclerotic lesion development in vaccinated animals.

Groups of C57BL/6 mice were immunized i.n. with PBS (mock vaccinated), IL-12, rCPAF, and rCPAF+IL-12, and followed by *C. pneumoniae* challenge. As shown in Figure 4a, all groups displayed comparable serum cholesterol levels on day 100, indicating there were no detected differences in dietary consumption or lipid metabolism. Importantly, as shown in Fig 4b, rCPAF+IL-12-immunized mice displayed significantly reduced atherosclerotic pathology in aortas on day 100 when compared to mock- or IL-12 immunized animals, and at a level comparable to that in mock (PBS)-immunized, mock (PBS)-infected animals fed the high-fat diet. On day 150 after *C. pneumoniae* challenge, rCPAF and rCPAF+IL-12 immunized mice displayed significant reduction in atherosclerotic pathology when compared to mock (PBS)- or IL-12-immunized animals. The addition of IL-12 as adjuvant appeared to enhance protective immunity induced by rCPAF alone in the early stages (day 100 after challenge), not at later time-points (day 150 after challenge) in atherosclerotic disease progression. These results demonstrate that Ag-specific immune response against *C. pneumoniae* rCPAF provides significant protection against atherosclerotic pathology exacerbated by *Chlamydia pneumoniae* infection in high-fat diet-fed mice.

DISCUSSION

In this study, we have demonstrated that i.n. immunization with rCPAF induces a robust Ag-specific IFN- γ and TNF- α cellular cytokine and antibody response. The splenic Ag-specific IFN- γ response and IgG2c/IgG1 anti-CPAF antibody ratio is enhanced significantly by addition of IL-12 as adjuvant. Immunization with rCPAF+IL-12 also enhances clearance of

pulmonary *Chlamydia pneumoniae* at later time periods following infection, which correlates with induction of splenic Ag-specific IFN- γ response against CPAF following challenge. Importantly, rCPAF alone or rCPAF+IL-12 immunization provides significant protection from *C. pneumoniae*-induced exacerbation of atherosclerosis in high fat diet-fed C57BL/6 mice.

We have shown previously that immunization with rCPAF cloned from *C. trachomatis* serovar L2 genome, with IL-12 or CpG-ODN as adjuvant, can enhance clearance of *C. muridarum* and *C. trachomatis* infection in mice and guinea pigs, respectively^{2-4, 21-28}. We also have shown that rCPAF+IL-12 vaccination protects against *C. muridarum*-induced disease sequelae in the female mouse upper reproductive tract including hydrosalpinx formation and infertility^{3, 4, 27, 28}. However, these disease sequelae were not induced by *C. trachomatis* infection in mice or guinea pigs and therefore we have not been able to confirm the efficacy of the vaccine against the human pathogen. In this context, we noted that CPAF is a highly conserved chlamydial protein, and a strong association between *C. pneumoniae* and exacerbation of atherosclerosis in human individuals, and a cause-and-effect relationship in mice and rabbits has been demonstrated¹. We have now demonstrated that rCPAF+IL-12 vaccination significantly attenuates *C. pneumoniae*-induced exacerbation of atherosclerosis in the C57BL/6 male mouse model. Given our prior observation that immunization with rCPAF protects against *C. muridarum* sequelae in mice and *C. trachomatis* infection in the Guinea pig, a paradigm of rCPAF as a broadly protective antigen now exists. These results further provide compelling evidence for inclusion of CPAF into a multi-subunit vaccine against a wide range of *Chlamydia*-induced disease sequelae in humans.

METHODS

CPAF and adjuvant.

Recombinant CPAF (rCPAF) was prepared as described previously³. Briefly, rCPAF construct from *Chlamydia pneumoniae* AR39 genome (Genbank GCA_000091085.2) with a 6X histidine tag (His) were cloned into pET23 vectors, expressed in *Escherichia coli* (BL21), and purified using Ni-NTA agarose beads (Amersham Biosciences). The purified protein was probed with anti-His antibody using western blot, and identity further confirmed using protein analysis by mass spectrometry. Endotoxin levels were measured using endotoxin LAL assay kit (ToxinSensorTM, Genscript), and were found to be 2.66-2.93 EU ml⁻¹, well within the recommended <20 EU ml⁻¹ level for recombinant protein vaccines²⁹. Recombinant mouse IL-12 was obtained from R&D systems.

Bacteria

Chlamydia pneumoniae AR39 (from Dr. Lee Ann Campbell, University of Washington) was grown on confluent Hep-2 cell (ATCC® CCL-23TM) monolayers as described previously³⁰. Cells were lysed using a sonicator and elementary bodies purified on Renograffin gradients. Aliquots of bacteria were stored at -70°C in sucrose-phosphate-₂PO₄ 3.8mM, K₂HPO₄ 7.2mM, L-Glutamate 4.9mM, pH 7.2 buffer).

Animals

Male 4-6 week-old C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and all experiments described in this manuscript were approved by the Institutional Animal Care and Use Committee of Midwestern University. Mice were anesthetized (3% isoflurane), and immunized i.n. with rCPAF (15 µg) mixed with IL-12 (0.5 µg) adjuvant on days 0, 14, and 28 as described previously³. Mice were primed i.n. with 0.5 µg of IL-12 on day -1 and +1. Optimal doses for rCPAF and IL-12 administration were based on our previous findings³. At the end of the experiment, mice were euthanized using carbon di-oxygen inhalation followed by cervical dislocation.

Antigen-specific cytokine response

Antigen (Ag)-specific cytokine production from splenocytes was measured as described previously³. Fourteen days after the initial i.n immunization with rCPAF or PBS (mock), mice were euthanized. Single cell suspensions of splenocytes (10^6 cells per well) were stimulated in culture plates with 0.1 µg of rCPAF, the unrelated protein bovine serum albumin (BSA), or media (RPMI 1640, Corning™ 10040CM) alone, and incubated for 72 hr. Supernatants were collected and assayed for IFN-γ and TNF-α production by enzyme-linked immunosorbent assays (ELISA) using BD OptEIA™ kits (BD Pharmingen, San Diego, CA).

Humoral response

Twelve days after the final (day 28) immunization, sera from the animals were analyzed by ELISA as described previously³. Microtiter plates coated overnight with rCPAF ($5 \mu\text{g ml}^{-1}$) were probed with serial dilutions of serum were added to wells followed by either goat anti-mouse total Ab, IgG1, or IgG2c, conjugated to alkaline phosphatase (Southern Biotechnology Associates, AL). After addition of p-nitrophenyl phosphate substrate (Sigma, MO), absorbance at 405 nm was measured using a microplate reader (Biotek Instruments, VT). Reciprocal serum dilutions corresponding to 50% maximal binding were used to obtain titers.

C. pneumoniae infection and determination of lung bacterial burden

One month following the final vaccination, mice were anesthetized with isoflurane and challenged i.n. with 2×10^7 IFU per mouse *C. pneumoniae* in SPG buffer (20 µl/nostril). Mice were challenged again 2- and 4-weeks after first challenge. From the day of first challenge, a high-fat diet (HFD; 15% fat, 1.25% cholesterol and 0.5% cholate)³⁰ was initiated and continued until the end of the experiment. Lung bacteria burdens were evaluated on the days 3, 6, 9, and 12 after first challenge. Lung homogenates were serially diluted in SPG and 0.2 ml of each dilution was centrifuged ($500 \times g$, 1 h, 35°C) onto Hep-2 cell-cultures grown in coverslips in 24-well plates (Costar). Plates were incubated for 1 h at 37°C, before replacing the inocula with 2 ml of $0.7 \mu\text{g ml}^{-1}$ cycloheximide-containing medium. After 72 h of incubation, cells were fixed with methanol and probed with mouse anti-*Cpn* polyclonal antibody (generated in-house from sera of infected C57BL/6 mice), and stained using FITC-conjugated rabbit-anti-mouse secondary antibody. Chlamydial inclusions were counted by fluorescence microscopy.

Serum total cholesterol

Mice were anesthetized using 3% isoflurane and blood was collected from the submandibular vein. Total serum cholesterol levels were analyzed using a kit (Catalog # ab65390, Abcam, Cambridge, MA).

Assessment of atherosclerotic lesions in the aorta

Mice were anesthetized at day 100 or 150 following initial infection. After perfusion of heart with 10ml PBS containing 100 IU heparin followed by 10 ml 2% paraformaldehyde, the aortas were excised from the aortic arch to the iliac bifurcation. Adventitial fat was removed and specimens were fixed in 2% paraformaldehyde. Whole aortas were opened longitudinally and mounted *en face*, stained for lipids with Oil Red O. Images were acquired using a Bioreader-FZ-beta ELISPOT instrument (Biosys, Germany) with inverted optics. The ratio of the area covered by the atherosclerotic lesions to the total surface area of aortic tissue was quantified using ImageJ software (National Institutes of Health, USA). The percent lesion area was calculated using the formula: (pixels in oil red O-stained region) $\times$ 100/ (pixels in the entire surface area of aortic tissue) as previously reported ³⁰.

Statistical analyses

Statistical analyses were performed using Analysis of variance (ANOVA; Systat software). *P*-values $\leq$ 0.05 were considered significant. All experiments were repeated once to confirm findings.

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Figure 1. Antibody response to immunization.

Groups of 4–6-week-old male C57BL/6J mice ($n = 9$) were immunized with the indicated antigen with or without adjuvant on days 0, 14, and 28, and anti-CPAF total Ab, IgG2c, and IgG1 responses analyzed on day 40. The IgG2c/IgG1 ratio is also shown for rCPAF alone or rCPAF+IL-12 immunized groups. N/A- not applicable, as individual titers were minimal. Mean $\pm$ SD of reciprocal 50% binding antibody titers or IgG2c/IgG1 ratio are shown. * Significant ($P < 0.05$; ANOVA) between indicated group and PBS (mock), as well as indicated group and IL-12-immunized animals. Ψ Significant ($P < 0.05$; ANOVA) between indicated group and rCPAF immunized animals. Results are representative of two independent experiments.

Figure 2. Splenic CPAF-specific cytokine response following immunization.

Groups of 4–6-week-old male C57BL/6J mice ($n = 3$) were immunized with the indicated antigen with or without adjuvant on days 0 and splenic Ag-specific IFN- γ and TNF- α response was measured on day 14. Mean $\pm$ SD of the cytokine levels in each group is shown.

* Significant ($P < 0.05$; ANOVA) between indicated group and PBS (mock), as well as indicated group and IL-12-immunized animals. Ψ Significant ($P < 0.05$; ANOVA) between indicated group and rCPAF immunized animals. Results are representative of two independent experiments.

Figure 3. Chlamydial burden and CPAF-specific cytokine response *C. pneumoniae* challenge.

Groups of 4–6-week-old male C57BL/6J mice ($n = 12$) were immunized with the indicated antigen with or without adjuvant on days 0, 14, and 28. Once month after final immunization, mice were challenged i.n. with 2×10^7 IFU/mouse of *C. pneumoniae*. (a) Lung chlamydial burden was analyzed on days 3, 6, and 9 after challenge using 4 mice per time-point. Mean $\pm$ SD of chlamydial IFU/ml per group at each time point is shown. (b & c) Splenic CPAF-specific IFN- γ or TNF- α production was evaluated on day 3 (data not shown as cytokine levels were minimal), day 6 (b), and day 9 (c) following primary challenge. Mean $\pm$ SD of cytokine concentration is shown. * Significant ($P < 0.05$; ANOVA) between indicated group and PBS (mock), as well as indicated group and IL-12-immunized animals. Ψ Significant ($P < 0.05$; ANOVA) between indicated group and rCPAF immunized animals. Results are representative of two independent experiments.

Figure 4. Atherosclerotic pathology following intranasal *C. pneumoniae* challenge.

Groups of 4–6-week-old male C57BL/6J mice ($n = 16$) were immunized with the indicated antigen with or without adjuvant on days 0, 14, and 28. One month after final immunization, mice were challenged i.n. with 2×10^7 IFU/mouse of *C. pneumoniae*. One group ($n=8$) of mock (PBS) immunized mice was mock-challenged with PBS. **(a)** On day 100, serum cholesterol level was analyzed. Mean $\pm$ SD of cytokine concentration is shown. **(b)** On day 100 or 150, atherosclerotic pathology as evaluated. The number of mice analyzed in each group at a particular time-point is indicated over the X-axis. Results are displayed as a box-whisker plot with defined upper and lower quartiles, upper and lower extremes, and the median. * Significant ($P < 0.05$; ANOVA) between indicated group and PBS (mock), as well as indicated group and IL-12-immunized animals. Results are pooled from two independent experiments.