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

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*Chlamydia trachomatis* is the leading cause of sexually transmitted bacterial disease worldwide (8). Despite the availability of potent antimicrobial drugs, the majority of genital chlamydial infections are initially asymptomatic and thus not treated (8, 31). Left untreated, chlamydial infections induce immunopathology in the uterus and fallopian tubes, causing pelvic inflammatory disease and complications such as ectopic pregnancy and infertility (8, 25, 56, 57). Additionally, short-lasting natural immunity against chlamydial infections and nontreatment of affected sexual partners result in high reinfection rates (25). The incidence of genital tract chlamydial infections is highest in women at the peak of their reproductive life (28), and significant health care costs are associated with management of these infections and the resulting complications (31), underscoring the urgent need for an efficacious antichlamydial vaccine.

To this end, multiple vaccination strategies for protection against genital *C. trachomatis* infection have been evaluated. Immunization with inactivated chlamydial organisms or passive transfer of dendritic cells pulsed ex vivo with inactivated chlamydial elementary bodies has been shown to result in significant degrees of protection against genital *Chlamydia muridarum* challenge (20, 27). The chlamydial major outer membrane protein (MOMP), which is abundantly expressed on the bacterial surface, has been considered the most likely vaccine candidate and has been extensively studied (31). Recombinant MOMP, MOMP synthetic peptides, DNA vaccines encoding MOMP, and the passive transfer of MOMP-specific monoclonal antibodies have been evaluated and found to be only partially effective in protecting experimental animals from subsequent challenge (5, 11, 18, 19, 22, 36, 38, 39, 50, 53, 59, 60). More recently, refolding of MOMP to achieve the native conformation prior to immunization has been reported to effectively reduce vaginal bacterial shedding and infertility rates in...
mice after genital chlamydial challenge (37). Immunization with an anti-idiotypic antibody to the chlamydial exolipid antigen has been shown to induce partial protection against genital *C. trachomatis* challenge (58). Thus, only a limited number of candidate chlamydial antigens have been evaluated to determine their protective efficacies, and the efforts have focused primarily on surface-bound structural antigens. However, the genomic sequence of *C. trachomatis* indicates the potential of several other novel antigens, either structural or secreted, that have yet to be evaluated experimentally as vaccine candidates (31).

Chlamydial protease-like activity factor (CPAF) is a protein secreted into the host cytosol that degrades major histocompatibility complex transcription factors RFX-5 and USF-1, allowing evasion of immune recognition (61, 62, 63). CPAF also has been shown to degrade keratin-8, possibly allowing evasion of the chlamydial “inclusion” inside cells and the probable spread of the bacterium in the extracellular matrix (16). An active form of CPAF has been shown to be produced during infection with five different species of chlamydiae (17). *Chlamydia*-seropositive humans exhibit higher titers of serum antibodies against CPAF than against exposed surface proteins (i.e., MOMP and Hsp60), indicating the immunogenic dominance of this secreted protein (47, 48, 49). These results also support the hypothesis that CPAF is important in the chlamydial life cycle and the conclusion that this secreted protein should be evaluated as a potential vaccine candidate.

In this study, we examined the protective efficacy of intranasal (i.n.) immunization with recombinant CPAF (rCPAF) in combination with murine interleukin-12 (IL-12) against genital *C. trachomatis* mouse pneumonitis (recently designated *Chlamydia muridarum*) challenge. Intranasal vaccination with rCPAF plus IL-12 (rCPAF + IL-12 vaccination) induced robust cell-mediated and humoral immune responses and significantly reduced the time required for resolution of a genital chlamydial infection. Importantly, rCPAF + IL-12 vaccination reduced the incidence of oviduct dilatation and hydrodropsalpinx in challenged mice. The protective efficacy of rCPAF + IL-12 vaccination also was shown to be highly dependent on endogenous gamma interferon (IFN-γ) production. Taken together, these results demonstrate for the first time that a secreted chlamydial protein, CPAF, is a viable vaccine candidate that should be considered for induction of efficacious, antichlamydial immunity.

### MATERIALS AND METHODS

rCPAF and IL-12. rCPAF encoded by the *C. trachomatis* serovar L2 genome was cloned and expressed in a bacterial system as described previously (16). Briefly, rCPAF constructs cloned from the *C. trachomatis* L2 genome with a six-histidine (His) tag were cloned into pBAD vectors and expressed in *Escherichia coli* with isopropyl-β-D-thiogalactopyranoside (IPTG) as an inducer. The fusion protein was purified using Ni-nitrilotriacetic acid agarose beads (Amer sham Biosciences Corp.). The purified rCPAF was identified by Western blot analysis using a monoclonal anti-CPAF antibody (15). CPAF activity was determined by determining the ability to degrade transcription factor RFX-5 in a concentration-dependent fashion, using a cell-free degradation assay, as described previously (61). The purified rCPAF was used as a source of protein for all experiments.

A recombinant BA1 protein (designated HisBA-1) was cloned from the *Francisella tularensis* genome, similarly expressed with a six-histidine tag, and used as a specificity control in some experiments. Murine recombinant IL-12 was a generous gift from Wyeth (Cambridge, MA).

**Bacteria.** *Chlamydia muridarum* was grown on confluent HeLa cell monolayers. Cells were lysed using a sonicator (Fisher, Pittsburgh, PA), and elementary bodies were purified on Renografin gradients as described previously (64). Aliquots of bacteria were stored at −70°C in sucrose-phosphate-glutamine buffer. For some experiments, *C. muridarum* stocks were inactivated using UV light as described previously (47).

**Mice.** Four-week-old, female BALB/c mice were obtained from Charles River Laboratories (Bar Harbor, ME). Age-matched female BALB/c IFN-γ mice were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were housed and bred at the University of Texas at San Antonio and were given food and water ad libitum. Animal care and experimental procedures were performed in compliance with the Institutional Animal Care and Use Committee guidelines.

**Intranasal immunization procedures.** Intranasal immunization was performed as described previously (3). Briefly, mice were anesthetized i.n. with 3% isoflurane anesthesia system (Harvard, or bronchoalveolar lavage (BAL)) or vaginal lavage fluids were obtained and analyzed by an enzyme-linked immunosorbent assay (ELISA) as described previously (34). Microtiter plates (96 wells) were coated overnight with 5 μg rCPAF per well or with the same concentration of an unrelated antigen (hen egg lysozyme [HEL]), Helicobacter pylori outer membrane units (15 μg/mouse) in previous experiments (31). Serial dilutions of rCPAF (1 to 20 μg/mouse) were evaluated to determine their protective efficacies against genital *C. muridarum* challenge (data not shown).

**Antigen-specific splenocyte recall responses.** Splenocytes were removed 14 days after primary vaccination, and single-cell suspensions were prepared. Collected cells (10⁶ spleen cells/well) were incubated for 72 h with 1 μg rCPAF per well or with the same concentration of an unrelated antigen (hen egg lysozyme [HEL]), Helicobacter pylori outer membrane units (15 μg/mouse) in previous experiments (31). After washing, the anti-CPAF antibody (Stigma, St. Louis, MO) was added for color development, and the absorbance (optical density) at 405 nm was monitored using a Quant ELISA microplate reader (BioTek Instruments, Winooski, VT).

**Detection of antibody and isotype levels by ELISA.** Ten days following final immunization, animals were bled to obtain serum, or bronchoalveolar lavage (BAL) or vaginal lavage fluids were obtained and analyzed by an enzyme-linked immunosorbent assay (ELISA) as described previously (34). Microtiter plates (96 wells) were coated overnight with 5 μg rCPAF in sodium bicarbonate buffer (pH 9.5). Serial dilutions of serum or undiluted bronchoalveolar lavage fluid or vaginal lavage fluid were added to wells, followed by either goat anti-mouse total immunoglobulin, immunoglobulin G1 (IgG1), IgG2a, IgG2b, IgM, or IgA conjugated to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). After washing (Sigma Instruments), a 3,5-diaminobenzidine (DAB) substrate (Sigma, St. Louis, MO) was added for color development, and the absorbance (optical density) at 405 nm was monitored using a μQuant ELISA microplate reader (BioTek Instruments). Reciprocal serum dilutions corresponding to 50% maximal binding were used to obtain titers. Because of the small amounts of antibody in BAL and vaginal fluids and the large dilution involved in the lavage procedure, these samples were tested undiluted. No binding of immune serum was detected in plates coated with HEL or HisBA-1 or to UV-inactivated *C. muridarum*.

**Vaginal *C. muridarum* challenge and determination of bacterial shedding.** One month following the final vaccination, 6 to 10 animals were anesthetized using inhalational isofluorane (3%) and challenged intravaginally (i.vag.) with 5 × 10⁶ IFU of *C. muridarum* in 5 μl of sucrose-phosphate-glutamine buffer. Two doses of depo-provera (Pharmacia Upjohn, Kalamazoo, MI) were injected subcutaneously on days −10 and −3 before challenge. To monitor bacterial shedding, vaginal swabs were obtained on different days after vaginal challenge, as indicated below, and this was followed by plating of the swab material on HeLa cell monolayers grown on culture coverslips. Chlamydial inclusions were detected using a murine anti-Chlamydia genus-specific murine monoclonal primary antibody and goat anti-mouse IgG secondary antibody conjugated to Cy3 plus Hoescht nuclear stain. The average number of inclusions in five random microscopic fields was calculated for each animal for earlier times (until day 12 after challenge), and the average number of inclusions on an entire coverslip was calculated for each animal for later times (days 15 to 30 after challenge); the results were expressed as the average number of inclusions per animal group.

**Histology and staining.** Genital tracts were removed from mice at various times after challenge, fixed in 10% neutral formalin, and embedded in paraffin blocks. Serial horizontal sections (5 μm) were prepared and stained using hematoxylin and eosin. Stained sections were visualized using a Zeiss Axioskop 2
Plus research microscope, and images were acquired using an Axiocam digital camera (Zeiss, Thornwood, NY).

Histological scoring. Sections stained with hematoxylin and eosin were scored in blinded fashion by a trained pathologist using a scoring scheme modified from the scheme described by Rank et al. (43). Dilatation of oviducts was scored as follows: 0, no significant dilatation; 1, mild dilatation of a single cross section of oviduct; 2, one to three dilated cross sections of oviduct; 3, more than three dilated cross sections of oviduct; and 4, confluent pronounced dilatation of oviduct. Cellular parameters (levels of polymorphonuclear leukocytes [PMNs], mononuclear cells, and plasma cells) were individually scored as follows: 0, no significant infiltration; 1, infiltration at a single focus; 2, infiltration at two to four foci; 3, infiltration at more than four foci; and 4, confluent infiltration. The results are expressed as means ± standard errors of the means for scores from all animals in a group (n = 6 to 8).

Statistical analyses. For comparison of two groups, the Student t test (for normally distributed values) or the Mann-Whitney rank sum test (for values not distributed normally) was used to compare values for continuous variables. For experiments with four groups of animals, analysis of variance was used followed by a multiple comparison of means (Kruskall-Wallis test) was used. To analyze differences in the time required for clearance, the Kaplan-Meier test was used. Differences between groups were considered statistically significant if the P values were <0.05. All data shown below are representative of two to five independent experiments, and each experiment was analyzed independently.

RESULTS

Intranasal immunization with rCPAF plus IL-12 induces robust cellular Th1-type immune responses. It has been reported previously that cell-mediated immunity is a crucial component of protective immunity against Chlamydia (26, 45). Therefore, we examined whether vaccination with rCPAF plus IL-12 induces antigen-specific cell-mediated responses. Mice were vaccinated, and rCPAF-induced cytokine recall responses

![Graph A](image.png)  
**A** Cytokine recall responses after immunization. Animals (three mice/group) were treated i.n. with rCPAF+IL-12, rCPAF alone, IL-12 alone, or PBS (Mock). On day 14, animals were euthanized, and spleens were tested for rCPAF-induced IFN-γ and IL-4 production by ELISA. The asterisks indicate that there are significant differences in IFN-γ and IL-4 secretion between rCPAF+IL-12 immunization and rCPAF immunization (P < 0.05, as determined by Student’s t test). (B to D) Systemic and mucosal anti-rCPAF antibody (Ab) responses after immunization. (B) Animals (10 mice/group) were bled on day 40 after primary immunization. Serum anti-rCPAF antibody levels were analyzed by ELISA using rCPAF-coated microtiter plates. The data are means ± standard errors of the means for reciprocal serum dilutions corresponding to 50% maximal binding. (C) Bronchoalveolar lavage fluids were collected from animals on day 40 after primary immunization (six mice/group) and analyzed by ELISA. (D) Vaginal fluids were collected on day 40 after primary immunization (10 mice/group) and analyzed by ELISA. Means ± standard errors of the means are shown for all experiments. An asterisk indicates that there is a significant difference between rCPAF+IL-12- and rCPAF-immunized animals (P < 0.05, as determined by the Kruskall-Wallis test). The results are representative of the results of two to five independent experiments. O.D.(405 nm), optical density at 405 nm.
in splenocytes were analyzed 14 days postvaccination. As shown in Fig. 1A, antigen-specific IFN-γ production was significantly greater (P < 0.05) in spleen cells from rCPAF+IL-12-immunized animals than in spleen cells from mice immunized with rCPAF alone. Conversely, IL-4 production was reduced (P < 0.05) in rCPAF+IL-12-immunized mice compared to rCPAF-immunized animals. Splenocytes from mock-vaccinated (PBS) or IL-12-treated mice did not exhibit detectable cytokine induction. The specificity of the response against rCPAF was shown by the minimal induction of cytokine production in spleen cells stimulated with either HEL, UV-inactivated C. muridarum that did not express CPAF in the metabolically inactive elementary body stage, or an unrelated six-histidine-tagged protein cloned from F. tularensis (HisBA-1). These results indicate that i.n. rCPAF+IL-12 vaccination induces a strong Th1-biased antigen-specific cellular immune response.

Intranasal rCPAF+IL-12 immunization induces systemic and mucosal antibody responses. The humoral response to rCPAF immunization was examined on day 40 after the initial i.n. immunization. Intranasal vaccination induced a robust serum antibody response that included rCPAF-specific total antibody, IgG2a, IgG2b, and IgG1 isotypes (Fig. 1B). Specifically, rCPAF+IL-12-immunized mice exhibited significantly enhanced (P < 0.05) titers of anti-rCPAF total antibody and IgG2a antibody but not of IgG2b and IgG1 antibodies compared to animals vaccinated with rCPAF alone. Since mucosal antibodies are important in protection against pathogens (6, 9, 51, 54, 55), induction of an antibody response at local inductive sites was measured in BAL (Fig. 1C) and vaginal (Fig. 1D).

<table>
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<tr>
<th>TABLE 1. Percentage of immunized animals shedding Chlamydia after genital challenge&lt;sup&gt;a&lt;/sup&gt;</th>
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<sup>a</sup> Animals (10 mice/group) were treated with three doses of rCPAF+IL-12, rCPAF, IL-12, or PBS (Mock). One month following the final vaccination, mice were challenged i.vag. with 5 × 10⁴ IFU of C. muridarum. On the days following challenge indicated, chlamydial shedding was measured. There were significant differences in the time required for resolution of infection between rCPAF+IL-12-immunized mice and all other experimental groups (P < 0.0002, as determined by the Kaplan-Meier test). The results are representative of the results of three independent experiments.
fluids collected from immunized animals. rCPAF+IL-12-immunized animals exhibited significantly higher levels ($P < 0.05$) of anti-rCPAF total antibody, IgG2a, and IgA in BAL fluids than animals immunized with rCPAF alone exhibited (Fig. 1C). Similarly, the rCPAF-specific total antibody, IgG2a, IgG1, and IgA titers in vaginal fluids of rCPAF+IL-12-vaccinated animals were significantly higher ($P < 0.05$) than the titers in vaginal fluids of mice that received rCPAF alone (Fig. 1D). Mock-vaccinated or IL-12-treated animals did not exhibit antigen-specific antibody responses in the serum or in respiratory and vaginal fluids. The serum antibody levels on days 14 and 28 (data not shown) exhibited comparable trends, with increased titers of specific antibodies in rCPAF+IL-12-treated animals, although the titers were lower than the titers at 40 days after the first immunization. Furthermore, no serum antibody responses against HEL, HisBA-1, or UV-inactivated $C. muridarum$ were found in any treatment group, clearly indicating the specificity of the immune responses generated by rCPAF. These results indicate that rCPAF+IL-12 vaccination enhances the resolution of a genital $C. muridarum$ infection. The protective efficacy of i.n. rCPAF vaccination was examined by monitoring the shedding of chlamydiae after intravaginal challenge with $5 \times 10^4$ IFU $C. muridarum$. As shown in Fig. 2A, there was a significant re-
duction (≥0.5 log) in the number of chlamydiae recovered from animals vaccinated with rCPAF+IL-12 as early as 8 days postchallenge compared to the number of chlamydiae recovered from mock-immunized (PBS) or IL-12-treated animals. Moreover, 30% of the rCPAF+IL-12-vaccinated animals had successfully resolved the infection by day 12, 80% of the animals had successfully resolved the infection by day 15, and 100% of the animals had successfully resolved the infection by day 18 (Table 1). In contrast, mock-vaccinated (PBS) animals or animals that received rCPAF or IL-12 alone were still heavily infected at day 15 (Fig. 2A). Forty percent of the mice vaccinated with rCPAF alone had resolved the infection by day 18 after challenge, and 70% had resolved the infection by day 24 after challenge (Table 1). In comparison, 30% of mice treated with PBS or IL-12 alone were still actively shedding chlamydiae 30 days after the bacterial challenge. These results demonstrate the efficacy of rCPAF+IL-12 vaccination for enhancing the resolution of a genital chlamydial infection.

rCPAF+IL-12 vaccination induces protection against C. muridarum-induced upper genital tract pathology. To determine the effect of rCPAF+IL-12 vaccination on the development of inflammatory disease, gross and histopathological changes in the oviduct and mesosalpingeal tissues were monitored following C. muridarum challenge. Since rCPAF+IL-12 immunization exhibited the greatest efficacy for resolution of genital chlamydial infection (Fig. 2A and Table 1), the C. muridarum-induced pathology of rCPAF+IL-12-vaccinated animals and the C. muridarum-induced pathology of mock-vaccinated (PBS) animals were compared. Genital C. muridarum infections in BALB/c mice have been shown to characteristically induce development of hydrosalpinx as a gross pathological complication (31). As shown in Fig. 3A, gross examination on day 80 postchallenge showed that hydrosalpinx was present in oviducts of mock-vaccinated (PBS) animals (Fig. 3A, upper panel), compared to the apparent normal appearance in rCPAF+IL-12-immunized mice (3A, lower panel). Specifically, at 50 and 80 days postchallenge, hydrosalpinx was not apparent in any of the rCPAF+IL-12-immunized mice, whereas most (88% and 75%, respectively) of the mock-immunized mice exhibited this gross pathological finding (Table 2). The observed pathology was bilateral (62.5%) in most mock-immunized (PBS) animals, but it was unilateral in some mock-immunized (PBS) animals at day 50 (25% of the mice) and day 80 (12.5%) after challenge. Hydrosalpinx was not apparent in the mice until day 30 after challenge. Mice treated with IL-12 alone and challenged with C. muridarum had a frequency of hydrosalpinx development similar to that of challenged PBS-treated (mock-immunized) animals (data not shown).

Detailed histopathological comparisons of rCPAF+IL-12- and mock-immunized (PBS) animals after genital C. muridarum challenge were also performed. While most of the rCPAF+IL-12-immunized mice (Fig. 3B) had apparently normal oviducts, mock-immunized (PBS) animals developed characteristic dilated oviducts (Fig. 3B) by day 80 after challenge. In addition, there were normal-appearing fimbriae in rCPAF+IL-12-immunized animals, while mock-vaccinated animals exhibited fimbrial flattening. Tissue sections from individual mice in both groups were blind scored by a trained pathologist in order to evaluate the degree of oviduct dilatation. As shown in Fig. 3C, the scores for oviduct dilatation on days 50 and 80 postchallenge in rCPAF+IL-12-vaccinated mice (0.2 ± 0.2 and 0.3 ± 0.18, respectively) were significantly lower than the scores for mock-immunized (PBS) animals (2.3 ± 0.75 and 3.2 ± 0.4, respectively). Very little oviduct dilatation was detected in either group until day 30 after challenge. In addition, Masson trichrome staining of tissues revealed significantly reduced fibrosis in rCPAF+IL-12-immunized animals compared to the fibrosis in mock-immunized animals on day 80 postchallenge (Fig. 3C).

The infiltration of inflammatory cells into the oviduct and mesosalpingeal tissues also was scored and evaluated for rCPAF+IL-12- and PBS-immunized mice on various days after challenge (Fig. 3D). These analyses revealed that the numbers of PMNs in tissues from the two groups of animals were comparable up to day 18 after challenge. However, on days 30 and 50 after challenge, the scores for the PMNs infiltrates in rCPAF+IL-12-vaccinated mice were significantly reduced (0.12 ± 0.1 and 0.1 ± 0.1, respectively) compared to the scores for the mock-immunized (PBS) animals (1.56 ± 0.17 and 1.6 ± 0.18, respectively). No significant PMN infiltration was detected in either group on day 80 postchallenge. The numbers of infiltrating mononuclear and plasma cells in rCPAF+IL-12-immunized animals also were significantly reduced compared to the numbers of these cells in mock-immunized animals from day 18 to day 80 postchallenge (Fig. 3D). Mice treated with IL-12 alone displayed degrees of oviduct dilatation and cellular infiltration similar to the degrees observed for challenged PBS-treated animals, whereas nonchallenged PBS-treated animals did not exhibit pathological changes at any of the observation times (data not shown). These results demonstrate that rCPAF+IL-12 vaccination reduces the pathology and duration of inflammatory cellular infiltration into the oviduct and mesosalpingeal tissues after genital C. muridarum challenge.

IFN-γ is important in mediating rCPAF+IL-12-induced genital protection. The role of IFN-γ in rCPAF+IL-12-mediated protection was examined using mice deficient in endogenous IFN-γ production (IFN-γ−/− mice). Vaccinated IFN-γ−/− mice began resolving the infection as early as 9 days
postchallenge (Fig. 4A and Table 3). By 9 and 12 days postchallenge, some vaccinated IFN-γ+/+ mice (33.3% and 50%, respectively) had completely resolved the infection, but no IFN-γ−/− mice (0%) had completely resolved the infection. On day 18 postchallenge, 100% of rCPAF+IL-12-vaccinated IFN-γ+/+ mice had resolved the infection, in contrast to only 16.7% of vaccinated IFN-γ−/− animals. As late as 50 days postchallenge, 50% of vaccinated IFN-γ−/− mice were still shedding low numbers of bacteria. In contrast, mock-immunized IFN-γ−/− mice completely resolved the infection by day 50 postchallenge, while a majority of similarly treated IFN-γ−/− mice (66.7%) were still shedding chlamydiae 50 days postchallenge, in agreement with previous studies (40). Moreover, the induction of rCPAF-specific antibody responses in vaccinated IFN-γ−/− mice (Fig. 4B) was comparable to that in similarly treated IFN-γ+/+ mice, suggesting that the absence of IFN-γ in these animals did not completely inhibit development of a humoral immune response after immunization.

The gross pathology and histopathology of the IFN-γ−/− and IFN-γ+/+ animals were also analyzed on day 80 after challenge. This time was chosen for these analyses based on previously described findings (31) and our previous experiment with BALB/c animals (Fig. 3), in which hydrosalpinx formation was not evident until day 50 after challenge and progressively increased until at least day 80 after challenge. As shown in Table 4, hydrosalpinx was not apparent in rCPAF+IL-12-immunized IFN-γ−/− mice (0%) but was apparent in similarly treated IFN-γ−/− mice (100% bilateral). Oviduct dilatation also was minimal in rCPAF+IL-12-immunized IFN-γ−/− mice (scores, 0.9 ± 0.3) compared to similarly treated IFN-γ+/+ mice.

FIG. 4. IFN-γ is required for resolution of genital tract infection in rCPAF+IL-12-immunized animals. Groups of IFN-γ+/+ and IFN-γ−/− mice (six mice/group) were immunized with three doses of rCPAF+IL-12 or PBS (Mock), rested for 1 month, and challenged i.vag. with 5 × 10^4 IFU of C. muridarum. Chlamydial shedding was measured on the days following challenge indicated. (A) Numbers of chlamydial IFU recovered from vaginal swabs on the days after genital challenge indicated. Each symbol represents an individual animal. The number(s) of animals in each group that had resolved the infection is indicated on the x-axis. An asterisk indicates that there is a significant difference between the groups indicated (P < 0.05, as determined by the Kruskall-Wallis test). (B) Serum anti-rCPAF antibody responses in immunized mice. Animals (six mice/group) were bled on day 40 after primary immunization, and sera were analyzed by an ELISA. The data are means ± standard errors of the means for reciprocal serum dilutions corresponding to 50% maximal binding. The results are representative of the results of two independent experiments. Ab, antibody.
mice (scores, 3.7 ± 0.33) (Fig. 5A). All rCPAF+IL-12- and mock-vaccinated (PBS) IFN-γ−/− mice displayed bilateral hydrosalpinx and confluent oviduct dilatation (scores, 3.7 ± 0.3 and 3.8 ± 0.2, respectively), indicating that there was no protective effect in vaccinated IFN-γ−/− animals. Additionally, cellular infiltration into the oviduct and mesosalpingeal tissues was examined on day 80 postchallenge (Fig. 5B). Fewer mononuclear cells were present in the oviduct and mesosalpingeal tissues of vaccinated IFN-γ−/− mice (scores, 1.5 ± 0.2) than in the oviduct and mesosalpingeal tissues of similarly treated IFN-γ−/− mice (scores, 2.25 ± 0.36), while both groups had minimal numbers of plasma cells and PMNs in their tissues. The cellular infiltration data for mock-immunized IFN-γ+/+ and IFN-γ−/− mice were comparable. In agreement with the previous experiment with BALB/c mice, rCPAF+IL-12-vaccinated IFN-γ−/− mice exhibited significantly reduced levels of hydrosalpinx, oviduct dilatation, and mononuclear and plasma cell infiltration compared to the levels in mock-vaccinated (PBS) IFN-γ+/+ animals. These results collectively suggest that rCPAF+IL-12-mediated protection is highly dependent on induction of endogenous IFN-γ production.

**DISCUSSION**

The incidence of genital chlamydial infection has risen over the last decade despite the availability of potent antimicrobial drugs, underscoring the importance and need for an efficacious vaccine (8). The chlamydial MOMP has been the focus of most current vaccine research. However, MOMP administration results in various degrees of protective immunity and is problematic due to native conformation and protein folding considerations (31, 37). Therefore, identification of other putative vaccine candidates that are used alone or in conjunction with MOMP against genital chlamydial infections is needed. CPAF is a secreted protein of *Chlamydia* that may be important for intracellular growth, as well as for evading host immune responses (16, 61). Furthermore, *Chlamydia*-seropositive humans exhibit high titers of anti-CPAF antibodies (47, 48), suggesting that vaccination strategies using CPAF may be beneficial for eliciting protective immunity against *Chlamydia*. In this study, we showed that i.n. immunization with rCPAF+IL-12 induces a robust Th1 cellular and humoral immune response and leads to accelerated resolution of genital *C. muridarum* infection in mice. rCPAF+IL-12 vaccination also greatly reduced the incidence of hydrosalpinx and oviduct dilatation, which are complications of genital infection. The protective effects of rCPAF immunization were shown to be highly dependent on endogenous IFN-γ production.

rCPAF+IL-12-immunized mice exhibited significantly reduced vaginal bacterial shedding, beginning as early as 8 days postchallenge, compared to mock-immunized (PBS) animals. In addition, the infection was completely resolved at a significantly (*P = 0.0002*) earlier time in rCPAF+IL-12-immunized mice (days 12 to 15 postchallenge) than in mock-immunized animals (days 24 to 30 postchallenge). The fact that the resolution in rCPAF+IL-12-vaccinated animals was faster but the animals were not resistant to infection suggests that cellular immunity, not neutralizing antibodies, may be important in mediating the protection. In this study, splenocytes from rCPAF+IL-12-immunized mice exhibited increased IFN-γ production along with concurrent minimal IL-4 production following in vitro rCPAF stimulation. Importantly, the protective effects of rCPAF+IL-12 vaccination were highly dependent on endogenous IFN-γ production. Specifically, vaccinated IFN-γ−/− mice exhibited reduced bacterial shedding (≥0.5 log) as early as 3 days postchallenge and at all subsequent times compared to the bacterial shedding in similarly treated IFN-γ−/− animals. Vaccinated IFN-γ−/− mice completely resolved the infection by day 15 postchallenge, whereas resolution was significantly delayed in vaccinated IFN-γ−/− animals, with 50% of the animals still shedding chlamydiae as late as 50 days postchallenge. Thus, despite relatively small differences in the bacterial burden early in the course of infection (until day 12 after challenge), vaccinated IFN-γ−/− mice exhibited acce-

### Table 3. Percentage of immunized IFN-γ−/− animals shedding *Chlamydia* after genital challenge

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<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>rCPAF+IL-12 IFN-γ−/−</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>83.3</td>
<td>83.3</td>
<td>66.7</td>
<td>66.7</td>
<td>66.7</td>
<td>66.7</td>
<td>50</td>
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</tbody>
</table>

* Groups of IFN-γ+/+ and IFN-γ−/− mice (six mice/group) were immunized with three doses of rCPAF+IL-12 or PBS (Mock), rested for 1 month, and challenged i.vag. with 5 × 10⁴ IFU of *C. muridarum*. Chlamydial shedding was measured on the days following challenge indicated. There were significant differences in the time to resolution of infection between rCPAF+IL-12-immunized IFN-γ−/− mice and all other experimental groups (*P < 0.0001*, as determined by the Kaplan-Meier’s test). The results are representative of the results of two independent experiments.

### Table 4. IFN-γ is required for prevention of hydrosalpinx development in *Chlamydia*-challenged CPAF+IL-12-immunized animals

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Mice</th>
<th>% of mice developing hydrosalpinx (n = 6) on day 80 after <em>C. muridarum</em> challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bilateral</td>
</tr>
<tr>
<td>Mock IFN-γ+/+</td>
<td>66.7</td>
<td>16.67</td>
</tr>
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<td>Mock IFN-γ−/−</td>
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</tr>
<tr>
<td>rCPAF+IL-12 IFN-γ+/+</td>
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<td>0</td>
</tr>
<tr>
<td>rCPAF+IL-12 IFN-γ−/−</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

* Animals (eight mice/group) were immunized with three doses of rCPAF+IL-12 or PBS (Mock), rested for 1 month, and subsequently challenged i.vag. with 5 × 10⁴ IFU of *C. muridarum*. On day 80 after challenge, animals (eight mice/group) were euthanized, and tissues were collected and used for analyses. The results are representative of the results of two independent experiments.
Act on FcR receptors (FcR) has been demonstrated for resolution of secondary chlamydial genital infections but not for resolution of primary chlamydial genital infections (29, 32, 33, 42). Anti-chlamydial IgG2a and IgA antibodies also have been shown to be important in resolution of genital infections (29, 32, 33, 42). Anti-CPAF antibody in CHI253 has been demonstrated to occur via separate host-specific mechanisms involving indoleamine dioxygenase in human cells and p47 GTPases in murine cells (35).

Intranasal rCPAF vaccination also induced a robust humoral response in systemic and mucosal compartments and specifically increased the levels of anti-CPAF IgG2a and IgA. Since CPAF may be important for evasion of immune recognition, neutralization of its activity may be beneficial to the host. To this end, Sharma et al. (47, 48) identified high titers of anti-CPAF antibody in Chlamydia-seropositive humans, including antibodies that neutralize the proteolytic activity of CPAF. Such neutralizing antibodies against CPAF have yet to be identified in mice. Moreover, CPAF+IL-12-vaccinated B-cell-deficient mice were able to resolve a genital chlamydial infection (A. Murthy and B. Arulananadam, unpublished observations), suggesting that humoral responses may not be absolutely required for CPAF-mediated protective immunity. However, an important role for B cells, antibodies, and Fc receptors (FcR) has been demonstrated for resolution of secondary chlamydial genital infections but not for resolution of primary chlamydial genital infections (29, 32, 33, 42). Anti-chlamydial IgG2a and IgA antibodies also have been shown to act on FcR+ dendritic cells but not on FcR+ dendritic cells in vitro, enhancing the activation of Th1 responses (30). Additionally, the passive transfer of monoclonal IgA against MOMP has been shown to enhance resistance and to reduce the intensity and duration of C. muridarum infection (47).

Although resolution of infection is an important component of an effective antichlamydial vaccine, the principal issue with chlamydial infections is the development of pelvic inflammatory disease and complications such as ectopic pregnancy and infertility. In our study, the incidence of hydrosalpinx and dilatation of oviducts after i.vag. C. muridarum challenge was significantly reduced in rCPAF+IL-12-vaccinated mice compared to mock-immunized animals. Challenged rCPAF+IL-12-vaccinated mice also exhibited significantly lower levels of fibrosis than mock-immunized animals. The mechanical obstruction caused by the ensuing fibrosis and toxic effects of the hydrosalpinx fluid on the ovum have been implicated in the development of infertility (1, 2, 46). The earlier resolution of infection in rCPAF+IL-12-vaccinated animals (days 12 to 15 after challenge) than in mock-immunized (PBS) animals (day 30 after challenge) could account for the significant prevention of pathological consequences. Thus, 14-day doxycycline therapy has been shown to completely prevent the development of hydrosalpinx in C. muridarum-infected mice when treatment was initiated on days 1, 3, and 7 postchallenge but not when treatment was initiated on day 10 postchallenge (52), indicating the effect of an extended bacterial load on pathological consequences. In addition, hydrosalpinx was not apparent in vaccinated IFN-γ+/+ mice (none of six animals examined) but was apparent in vaccinated IFN-γ−/− animals (all six animals examined; bilateral). Also, the incidence of fibrosis was significantly lower in vaccinated IFN-γ+/+ animals than in vaccinated IFN-γ−/− animals. Thus, IFN-γ has been shown to limit excessive inflammatory pathology associated with intracellular bacterial infections (10). Therefore, vaccination-induced antigen-specific IFN-γ responses not only are important for resolution of infection but also are an essential component of protective immunity against disease pathology.

rCPAF+IL-12-vaccinated animals also had lower frequen-
cies of PMNs, mononuclear cells, and plasma cells after genital chlamydial challenge than mock-immunized (PBS) animals had. Notably, at days 30 and 50 postchallenge, the number of infiltrating PMNs was lower in rCPAF+IL-12-immunized mice than in mock-immunized (PBS) animals. Neutrophil-generated inflammatory products, such as matrix metalloproteinase-9, have been implicated in the development of oviduct pathology and infertility (4, 25, 41). Therefore, reduction of PMN infiltration into the oviductal and mesosalpingeal tissues at an earlier point during the course of chlamydial infection also might account for the observed protection against upper genital tract pathology in vaccinated mice. The protection against pathological events in rCPAF+IL-12-vaccinated animals provides only indirect evidence in support of preservation of fertility. Determination of oviduct patency and fertility studies are necessary to determine the reproductive outcome of this vaccination regimen. Additionally, differences in mouse strains have been shown to affect the pathological outcome of genital chlamydial infections (14). Since this study was restricted to BALB/c mice, further studies using mice having different genetic backgrounds and this vaccination regimen are needed.

An important observation from our study is that immunization with recombinant CPAF from C. trachomatis serovar L2 provided protection against C. muridarum challenge. In fact, the amino acid sequences of CPAF from serovar L2 and C. muridarum exhibit significant (82%) identity (17). In addition, monoclonal antibodies raised against serovar L2 CPAF have been shown to recognize CPAF from either serovar L2 or C. muridarum (17), indicating that there is a high degree of immunological cross-reactivity. We also have recently detected CPAF in situ by immunohistochemistry within C. muridarum-infected genital tract epithelial cells using polyclonal mouse immune serum generated against CPAF from serovar L2 (Murthy and Arulandam, unpublished observations). Together, these results suggest the likelihood of a conserved epitope(s) on CPAF that is involved in protective immunity. Since there are 15 serovars of C. trachomatis that affect humans, a vaccine that provides cross-protection against multiple serovars would be beneficial. MOMP vaccines are homotypic and may not be ideal candidates (31), while the recently identified C. trachomatis polymorphic membrane protein-D is a species-common pan-neutralizing antigen and thus may also be a strong candidate to achieve broad-spectrum protection against chlamydial infections (13).

To our knowledge, here we provide the first evidence of an effective vaccination strategy using a secreted protein from Chlamydia. Contrary to the popular belief that a surface-exposed chlamydial protein would be an ideal vaccine candidate, we have demonstrated that CPAF, a secreted protein, can generate significant immune protection against genital chlamydial challenge. Intracellular bacteria, such as Chlamydia and Mycobacterium, are confined within the phagosome and physically exclude structural components from host immune recognition. However, products such as CPAF are secreted into the host cytosol and presumably escape into the extracellular compartment by exocytosis or upon rupture of infected cells. Therefore, CPAF should be readily available for processing and antigen presentation to CD4⁺ T cells via the major histocompatibility complex class II pathway. This conclusion is supported by the fact that intraperitoneal anti-CD4⁺ antibody treatment abrogates the protective effects of rCPAF+IL-12 vaccination (33a). A similar approach using bacillus Calmette-Guérin (Mycobacterium bovis BCG) expressing the Mycobacterium tuberculosis 30-kDa major secretory protein has been shown to induce more robust protective immunity than BCG alone induces against subsequent challenge with virulent organisms (21). The results of our study provide further support for using secreted products as candidate vaccines for intracellular bacteria.

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REFERENCES


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