We have comprehensively demonstrated using the mouse model that intranasal immunization with recombinant chlamydial protease-like activity factor (rCPAF) leads to a significant reduction in bacterial burden, genital tract pathology and preserves fertility following intravaginal genital chlamydial challenge. In the present report, we evaluated the protective efficacy of rCPAF immunization in guinea pigs, a second animal model for genital chlamydial infection. Using a vaccination strategy similar to the mouse model, we intranasally immunized female guinea pigs with rCPAF plus CpG deoxynucleotides (CpG; as an adjuvant), and challenged intravaginally with Chlamydia trachomatis serovar D (CT-D). Immunization with rCPAF/CpG significantly reduced vaginal CT-D shedding and induced resolution of infection by day 24, compared with day 33 in CpG alone treated and challenged animals. Immunization induced robust anti-rCPAF serum IgG 2 weeks following the last immunization, and was sustained at a high level 4 weeks post challenge. Upregulation of antigen-specific IFN-γ gene expression was observed in rCPAF/CpG-vaccinated splenocytes. Importantly, a significant reduction in inflammation in the genital tissue in rCPAF/CpG-immunized guinea pigs compared with CpG-immunized animals was observed. Taken together, this study provides evidence of the protective efficacy of rCPAF as a vaccine candidate in a second animal model of genital chlamydial infection.

Immunology and Cell Biology (2017) 95, 454–460; doi:10.1038/icb.2016.122

Chlamydia trachomatis is the leading cause of bacterial sexually transmitted diseases worldwide.1 When left untreated, it leads to chronic inflammatory conditions, including pelvic inflammatory disorder, ectopic pregnancies and infertility.2–5 Given the asymptomatic nature of infection in a high proportion of affected individuals, and the intracellular persistence of the organism, recurring infections and chronic disease lead to significant health care costs.5–6 Although the infection can be treated with antibiotics,7 preventive intervention by vaccination is considered the most effective measure to control chlamydial sexually transmitted diseases.

Currently, there is no licensed vaccine against Chlamydia spp.; however, our laboratory has extensively demonstrated that immunization with recombinant chlamydial protease-like activity factor (rCPAF) is highly effective in protection against subsequent challenge in the mouse model.8–16 Specifically, intranasal (i.n.) vaccination with rCPAF, with murine recombinant IL-12 or CpG deoxynucleotides as adjuvant, protects against subsequent genital chlamydial challenge and reduces the incidence of hydrosalpinx development in the upper reproductive tract.10,11 Moreover, rCPAF vaccination preserved fertility in mice repeatedly challenged with Chlamydia.15 Protection by rCPAF vaccination has been found to be mediated via IFN-γ secreting antigen-specific CD4+ T cells and antibodies.11,12 Although the mouse model is widely used for Chlamydia studies,17–19 the availability of other models, such as guinea pigs, pigs, minipigs, sheep, cattle and macaques,20–23 to study chlamydial pathogenesis and vaccine strategies is highly beneficial. Guinea pigs serve as a translation model between mice and human Chlamydia studies as they have a reproductive physiology and estrous cycle (15–17 days) similar to that of humans.20,24 In addition, certain guinea pig strains are outbred (as used in our study) and may add to understanding the genetic basis of differences in anti-chlamydial immunity in human cohorts. To date, guinea pigs have been used to evaluate the protective efficacy of recombinant major outer membrane protein against genital chlamydial infection, where reduction in genital pathology following Chlamydia challenge is associated with systematic and mucosal antibody production.25–27 Furthermore, our recent report on the regulation of guinea pig-specific genes in vaccinated animals using a 96-gene transcriptome array has advanced our understanding of guinea pig immune responses to vaccination and infection.28 In that report, we found that intranasal inoculation/vaccination with Chlamydia caviae elementary bodies induced robust neutralizing antibodies and regulated Th1- and Th2-related cytokine/chemokine genes, including IFN-γ,28 which has been shown...
to play a major role in the control of chlamydial infection in mice.8,29,30

Although C. caviae is the naturally infecting strain in guinea pigs and has been extensively used to study chlamydial infection in the guinea pig model,26,28 de Jonge et al., has developed the human C. trachomatis L2 serovar D and E infection model in guinea pigs and reported development of upper genital tract pathology comparable to human disease.31 The rCPAF vaccine extensively characterized by our group in the mouse model is derived from the human C. trachomatis L2 serovar and mouse C. muridarum strain, respectively.32 However, this rCPAF has only 54% identity to C. caviae CPAF protein, therefore, we utilized the newly developed guinea pig CT-D infection model31 to examine the efficacy of rCPAF as a vaccine in this second animal model.

RESULTS

Resolution of genital C. trachomatis challenge in guinea pigs immunized with rCPAF

Using a vaccination regimen similar to the one used for mice studies,10,11,16 we evaluated the protective efficacy of rCPAF in guinea pigs. In this study, we used CpG-10109 and a vaccination dosage based on other guinea pig reports25,33,34 and pilot studies in our laboratory. As shown in Figure 1a, CpG (mock) immunized guinea pigs displayed higher levels of chlamydial shedding initially, which then progressively decreased with complete clearing of the infection (no detectable bacterial shedding) by day 33. In contrast, guinea pigs i.n. inoculated with 10⁵ IFUs live CT-D (serves as positive control for immunization) exhibited significant reduction of bacterial shedding on days 3 and 6 post challenge with complete clearance on day 9. Immunization with rCPAF+CpG resulted in a significant reduction in chlamydial shedding compared with those receiving CpG alone as early as day 3 post challenge. The bacterial shedding profile displayed a significant reduction in CT-D- and rCPAF+CpG-immunized guinea pigs when compared with CpG-mock-immunized animals (Figure 1b). Also as shown in Figure 1c, the area under the curve showed a significant reduction in bacterial burdens in CT-D- and rCPAF+CpG-immunized guinea pigs compared with CpG-mock-immunized guinea pigs (Figure 1c). Furthermore, 40% of the rCPAF-immunized guinea pigs resolved infection on day 21 and all animals cleared infection by day 24; whereas, all CpG-mock-immunized animals still exhibited chlamydial shedding on day 27 and ultimately resolved the infection by day 33 (Figure 1d).

rCPAF/CpG vaccination induced antibody production in guinea pigs

Using cyclophosphamide treatment to preferentially suppress humoral, but not cell-mediated, immunity, Rank et al. have demonstrated that a humoral response is essential for guinea pigs to resolve primary35 and secondary36 C. caviae genital infections. To assess humoral responses induced by vaccination and challenge, we collected sera from immunized animals 2 weeks before challenge and 4 weeks post challenge and measured antibody reactivity. Before the bacterial challenge, CpG-vaccinated guinea pigs produced minimal serum antibody against rCPAF and EBs (UV-inactivated CT-D) as shown in Figure 2a. In contrast, rCPAF/CpG- and CT-D-vaccinated guinea pigs induced high levels of serum antibodies against rCPAF and CT-D, respectively. All animals, including CpG-vaccinated guinea pigs, mounted anti-CT-D humoral responses to i.vag. CT-D challenge (Figure 2b). Among the three study groups, CT-D i.n.-vaccinated guinea pigs produced the highest level of anti-CT-D antibody pre and post challenge. Serum anti-CT-D antibody production also increased in CpG and rCPAF/CpG-vaccinated animals after CT-D challenge (Figure 2b).

Figure 1 Vaccination enhanced chlamydial clearance from the guinea pigs genital tract. Groups (n=5) of guinea pigs were immunized i.n. with rCPAF/CpG or CpG alone (mock) and boosted twice at 2-week intervals. Another group (n=5) of guinea pigs received one i.n. dose of live C. trachomatis serovar D EBs (CT-D; 1×10⁵ IFUs). One month after the final immunization, guinea pigs were challenged i.vag. with 10⁵ IFU CT-D. Chlamydial shedding was monitored every third day post challenge until day 36 and presented as mean ± s.d. for each group at each time point. *Significant reductions (P<0.05; one-way ANOVA) in bacterial shedding by (a) bar graph and (b) overall area and (c) area under the curve between the indicated group and CpG-immunized (mock) guinea pigs are shown. (d) The number of guinea pigs shedding Chlamydia after genital challenge for each immunization group is summarized. (c) **Significant reduction (P=0.002), ANOVA with Tukey (b) between groups; (d) **Significant reduction (P=0.006 and P=0.0001, respectively, Fisher’s exact test) in number of rCPAF+CpG- and CT-D-immunized animals, compared with CpG-immunized animals, shedding chlamydia across all time-points evaluated. Results are representative of two independent experiments.

| Immunization   | D3  | D6  | D9  | D12 | D15 | D18 | D21 | D24 | D27 | D30 | D33 | D36 |
|----------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| CpG            | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 | 0/5 |
| rCPAF/CpG     | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 | 0/5 |
| CT-D**         | 5/5 | 5/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |

| Area under the curve | | | |
|----------------------| | | |
| CpG                  | 1.0 × 10⁴ | 1.0 × 10⁵ | 1.0 × 10⁶ |
| rCPAF/CpG           | 1.0 × 10⁴ | 1.0 × 10⁵ | 1.0 × 10⁶ |
| CT-D                 | 1.0 × 10⁵ | 1.0 × 10⁶ | 1.0 × 10⁷ |
rCPAF/CpG vaccination induced cellular IFN-γ response in guinea pigs

We have previously demonstrated that immunization with rCPAF induced a robust cell-mediated immune response with IFN-γ production and led to protection against genital chlamydial disease in mice. To evaluate the cell-mediated response by vaccination in guinea pigs, we collected three spleens from each study group 1 day before challenge. Splenocytes were stimulated in vitro with antigens rCPAF and UV-inactivated CT-D (UV-CT-D) or unstimulated (with medium) for 24 h and the gene expression of IFN-γ was assessed by quantitative real-time PCR. As shown in Figure 3, significantly higher transcript levels of IFN-γ were observed in splenocytes from the rCPAF/CpG group stimulated with rCPAF and in CT-D-immunized animals stimulated with UV-CT-D compared with their respective medium stimulations. IFN-γ gene transcript levels corresponded to elevated IFN-γ secretion in supernatants of splenocytes from rCPAF/CpG or CT-D-immunized guinea pigs stimulated with CpaF or CT-D compared with CpG-vaccinated guinea pigs (Supplementary Figure 1). In addition, TNF-α, a cytokine associated with its role in multifactorial Ag-specific T-cell-mediated protection, displayed increased/higher transcript levels (although not statistically significant) in splenocytes from the rCPAF/CpG group stimulated with rCPAF and in CT-D-immunized animals stimulated with UV-CT-D compared with their respective medium stimulations (Supplementary Figure 2). Taken together, these results indicate that both rCPAF/CpG and CT-D vaccination mounted modest increases (given the small numbers of animals/group) in Ag-specific Th1 cytokines such as IFN-γ which contribute to the cell-mediated immune response.

DISCUSSION

We have previously demonstrated that vaccination with rCPAF protects against genital chlamydial challenge by robust induction of cellular and humoral immune responses in the murine model. We now have demonstrated the protective efficacy of rCPAF in a second animal model, the guinea pig. Guinea pigs immunized with rCPAF/CpG exhibited lower levels of chlamydial shedding, a shortened duration of infection, and reduced upper genital tract pathology compared with CpG (mock)-immunized animals. This protection was correlated with robust antibody production and cellular IFN-γ induction in an antigen-specific manner. Also in line with our previous findings, we found a limited role for rCPAF-specific antibodies in the CT-D immunization regimen. Collectively, these results are consistent with the protective efficacy of rCPAF observed in the mouse model of chlamydial infection and further validate rCPAF role as a potential candidate to be used for the development of a licensed human Chlamydia vaccine.
Figure 4 Vaccination reduced histopathological lesions in indicated genital tracts following chlamydial challenge. Histopathology was assessed in naïve (uninfected/healthy) and *Chlamydia* challenged CpG-, rCPAF/CpG-, CT-D-vaccinated guinea pigs. The genital tract of each guinea pig was removed at day 65 post CT-D challenge, sectioned, hematoxylin and eosin stained and analyzed microscopically (original magnification of the images is x100 or x200). Histopathological injury in the genital tract of representative healthy and diseased guinea pigs was scored (a) and graphically represented for the respective group as a whole (b) for four distinct parameters (inflammatory cell infiltration, congestion, hemorrhage and edema). Obviously, the individual micrographs shown (a) represent the types of pathology observed in different regions of the genital tract of healthy and diseased animals, whereas the graph (b) summarizes these types of observations for all sections of all regions of all animals examined. The asterisk indicates significant reductions (*P*<0.05; Kruskal–Wallis test with Dunns post hoc test) between CT-D- or rCPAF/CpG-immunized groups in comparison to CpG group for the respective parameters.
In the current study, animals that received live i.n. *C. trachomatis* (CT-D) immunization exhibited rapid bacterial clearance and significant reduction in upper genital pathology following a secondary intravaginal chlamydial challenge. These results are consistent with those from the mouse model 

41, 43 and guinea pig vaccination studies 

25, 31 wherein, robust protection against genital chlamydial challenge is induced by live chlamydial EB immunization. Hydro-

salpinx (fluid-filled oviduct dilatations) are a characteristic feature of pathological sequelae following chlamydial infections in their respective hosts. Hydrosalpinx was not a common feature of the guinea pig infection model. However, histopathology was observed in the uterine tissue sections of the previously estradiol-treated, CpG-immunized and CT-D-challenged animals. Estradiol, not progesterone, treatment before chlamydial challenge in guinea pigs has been shown to increase the intensity and duration of *C. caviae* infection 

44 and is required to establish a sustained CT-D infection.

31 Although genital tract pathology induced by estradiol injection has been documented in guinea pigs 

45 the estradiol treatment following our study regimen (two 5 mg injections 1-week apart) in the absence of chlamydial infection did not cause visible inflammation 68 days after the second injection (data not shown) suggesting that hormonal treatment alone contributed minimally to upper genital pathology in our model. Furthermore, similar to other animal models, 

20 CT-D challenge in guinea pigs resulted in milder genital tract pathology and uterine inflammation than that seen with the natural pathogen *C. caviae*, as demonstrated by our group and others.

78, 46 In this context, *Chlamydia* have been shown to exhibit host tropism due to their adaptation to the restrictive influence of IFN-γ in respective hosts.

47 However, despite the reduced severity of pathological outcomes following CT-D infection in the guinea pig, this model produces sufficient bacterial shedding and pathology to evaluate the effects of experimental vaccines. To this end, studies by us and others using live or UV-EBs for vaccination have demonstrated significantly accelerated chlamydial clearance within 7 days post challenge 

41, 48 compared with single antigens, including rCPAF/CpG and MOMP, which result in protection 7–10 days later.

49 A correlation between early protection with neutralizing antibody induced by the live EB or UV-EB regimen may occur, whereas given that rCPAF is a RB-specific protein, anti-CPAF antibodies do not neutralize chlamydial infectivity, as demonstrated using B-cell deficient mice.

62, 13 We have previously characterized the efficacy of rCPAF cloned from *C. trachomatis* genome 

49 and further evaluated the effectiveness of this vaccine candidate (with only 54% amino acid identity to *C. caviae*) against a CT-D challenge in this study. Consistent with our previous findings in mouse studies, vaccination with rCPAF (derived from CT) in guinea pigs resulted in reduced chlamydial shedding and genital tract pathology, correlating with CPAF-specific immune responses. rCPAF is a dominant antigen expressed in CT-positive individuals.

50, 51 We have previously demonstrated that rCPAF immunization protects against a subsequent genital challenge in humanized HLA-DR4 transgenic mice, 

and it provides cross-serovar protection. 

49 The vaccination regimen conferred by rCPAF plus adjuvants (CpG or IL-12) exhibited greater protection that that observed by rCPAF vaccination alone.

15 Taken together, previous reports and this current study extend the importance of rCPAF as a vaccine candidate and highlight its use as a protective molecule in a second animal model against a human serovar of CT. Despite new and evolving information on cellular targets of CPAF, its role as a putative anti-*Chlamydia* vaccine candidate holds continued promise.

13, 34, 35 With rapid progression in genetic manipulation of *Chlamydia*, 

36 there also is enthusiasm for the development of a safe and more effective live-attenuated vaccine. In addition, recent studies have highlighted anti-chlamydial immune responses via protective tissue resident memory T-cell subsets in vaccinated mice, 

48 and B cells enhancing Ag-specific CD4+ T-cell priming upon infection.

56, 57 In the context of a future *Chlamydia* vaccine and a possible role for CPAF, there is no consensus as to whether the desired outcome is a reduction in infectivity/transmission or a reduction in the clinically relevant chronic pathologies. Along with the reduction in bacterial shedding (around second week), we have previously demonstrated superior protection against upper reproductive pathologies with the rCPAF regimen, 

11 including protection against infertility induced following repeated chlamydial challenge. 

15 Our findings, with regard to efficacy of rCPAF immunization, are in accordance with other reports.

58, 59 In addition, the concept that a reduction in shedding does not always correlate to protection against pathology is supported by O’Meara et al.

60 In summary, the guinea pig CT-D challenge model may be a useful for the evaluation of new and previously identified vaccine candidates. Vaccination with single protein antigens (such as CPAF and MOMP) may not be sufficient to generate desired protective efficacy in humans.

1, 4 To this end, effective vaccination strategies using multiple antigens, formulations and routes of delivery are critical.

61 Importantly, rCPAF with its ability to preserve fertility in mice, 

15 and reduce infection severity in guinea pigs has the potential to serve as an ideal antigen to be formulated into multivalent vaccines or be overexpressed in an attenuated live vaccine platform to prophylactically control human *Chlamydia* infection.

METHODS

Bacteria

*Chlamydia trachomatis* serovar D (from the Zhong Lab, UT Health Sciences Center, San Antonio, TX, USA) was grown on confluent HeLa cell monolayers. Infected HeLa cells were disrupted and vortexed with glass beads in a falcon tube for 5 min with 30 s intervals on ice followed by centrifugation for 10 min at 1200 rpm 4 °C. The supernatant was collected and spun at of 27 000 g for 1 h at 4 °C to obtain a bacterial pellet that was further purified on Renografin gradient as described earlier.

64 Purified elementary bodies were aliquoted and stored at −80 °C in sucrose-phosphate-glutamate buffer until use.

Guinea pigs

Dunkin Hartley strain guinea pigs (350–450 g) were purchased from Charles River Laboratories (Wilmington, MA, USA) and were housed in the AAALAC-accredited University of Texas at San Antonio Vivarium. Food and water were supplied *ad libitum* and all experimental studies were conducted humanely and followed the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol (IS0146) was approved for conducting this study by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas at San Antonio.

Immunization and challenge

Groups of guinea pigs (*n* = 5) were immunized i.n. on day 0 with 150 μg rCPAF (derived from human *C. trachomatis* serovar L2 with a 99% homology to serovar D) and expressed as fusion proteins in *Escherichia coli* as described previously 

5 and 25 μg of CpG nucleotides (CpG-101095, 52, 56), or CpG alone (mock), in 100 μl of sterile phosphate buffered saline. The dose of rCPAF was based on other reported protein vaccination studies using the guinea pig infection model.

25, 33, 34 Booster i.n. immunizations were provided on days 14 and 28 with 100 μg rCPAF and 25 μg of CpG. One month following the last booster, guinea pigs were challenged i.vag. with 1 × 10^5 IFU of CT-D re suspended in 50 μl sucrose-phosphate-glutamate buffer. Another group of guinea pigs was immunized i.n. with 1 × 10^5 IFUs of live CT-D once and rested.
for 8 weeks before challenge. To achieve a sustained CT-D infection in guinea pigs, all animals received a subcutaneous injection of 5 mg β-estradiol (Sigma, St Louis, MO, USA) in 100 μl sesame oil (Sigma) on days -10 and -3 before challenge as described by de Jonge et al.31 All guinea pigs were anesthetized with 3% isoflurane before immunization and challenge procedures. Following challenge, vaginal swabs were collected at a 3-day interval for 36 days from all groups of guinea pigs and plated onto HeLa cell monolayers to determine the chlamydial burden as described previously.28

Assay of humoral immune responses
Guinea pigs were bled from the lateral saphenous leg vein 15 days after the last booster (2 weeks before challenge) and 4 weeks post challenge as described.63 To measure antibody reactivity, microtiter plates were coated with 1 x 10^7 IFUs of UV-inactivated CT-D or 0.5 μg rCPAF and incubated at 4°C overnight. ELISA was performed using serial twofold diluted sera (starting with 1:100) as described28 to assess antibody reactivity. Following serial dilution of serum samples, plates were incubated for 2 h, followed by incubation with goat anti-guinea pig total IgG conjugated to horseradish peroxidase (ABD Serotec, Raleigh, NC, USA). Tetramethylbenzidine substrate was added and the absorbance quantified at 630 nm using a μQuant ELISA plate reader (BioTek Instruments, Winooski, VT, USA). End point titers of each serum for specific antigen(s) was determined by selecting the highest dilution factor at which the sample OD was greater than the average OD of six CpG-mock immunized pre-challenge sera, plus 2.2 s.d.s (to achieve 95% confidence as suggested by Frey et al.64), and OD value greater than 0.1.

Cytokine PCR and ELISA
Groups of guinea pigs (n = 3) were immunized i.n. with rCPAF/CpG or live CT-D as described above and killed 1 day before challenge to collect spleens aseptically for antigen-specific spleenocyte stimulation assay. Single spleenocyte cell suspensions were prepared (1 x 10^6 cells/well in 100 μl DMEM plus 10% FBS) and stimulated with 0.5 μg rCPAF, UV-inactivated CT-D (1 x 10^5 IFUs) or left unstimulated with media alone in a 96-well microtiter plate for 24 h. Following stimulation, cells were collected to assess INF-γ gene expression. Messenger RNA was isolated from stimulated spleenocytes as per the manufacturer’s instructions (RNasea mini kit, Qiagen, Valencia, CA, USA) and quantified using a Nanodrop spectrophotometer (Thermo Scientific NanoDropTM 1000, Waltham, MA, USA). RNA (0.5 μg) was used to generate cDNA using the Verso cDNA Synthesis (Thermo Scientific). The guinea pig IFN-γ (forward 5′-CCATCAAGGACAACTTATTAC-3′ and reverse 5′-TGAGGTTTTTGAATCAG-3′), TNF-α (forward 5′-GGAGAGCG AGTCTGCAAG-3′ and reverse 5′-GGCTGCTATCTGATTTGGAG-3′) and GAPDH (forward 5′-CTGGCCTATGAAAGG-3′, reverse 5′-GTTGATT CCACCTACATAC-3′) gene-specific primers were used to amplify gene transcripts from the synthesized cDNA. qRT-PCR was conducted under a previously optimized condition using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA)28 and IFN-γ and TNF-α mRNA levels were normalized to GAPDH mRNA and expressed as relative level to medium mock-stimulated samples using the comparative cycle threshold method.65 Supernatants from each condition were collected and stored at -80°C until further use. Guinea pig IFN-γ was assessed by a quantitative competitive immunoassay (NeoScientific, Cambridge, MA, USA) according to the manufacturer’s instructions. In brief, 100 μl of experimental supernatants or standards were co-incubated in wells with an IFN-γ conjugate. Binding of IFN-γ horseradish peroxidase was visualized by production of colorimetric reaction products that were quantitatively measured by absorbance using a μQuant ELISA plate reader (BioTek Instruments).

Genital tract pathology
Guinea pigs were killed and genital tract tissues were collected in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Histopathology imaging and pathological scoring were performed using a Carl Zeiss microscope as described previously.28 Pathology scores for each group was represented as mean and s.d. of each guinea pig in the respective group.

Animals and statistical analyses
For experiments, female guinea pigs were age matched and numbers/group was selected based on previous findings.11,28 All result data sets from experiments were included for analyses and were not excluded from the study. No randomization or blinding was used. GraphPad Prism 5 (La Jolla, CA, USA) was used to perform all statistical tests. P < 0.05 was considered statistically significant. One-way ANOVA or Kruskal–Wallis test with Dunns post hoc test was used for determining the protective efficacy of rCPAF vaccination compared with other treatment groups. Appropriate statistical tests are indicated in legends of respective figures. All data are representative of two independent experiments and each experiment was analyzed independently.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ACKNOWLEDGEMENTS
We thank Dr Roger Rank (Arkansas Children’s Hospital Research Institute) for his support in establishing the guinea pig model and insightful discussions. This work was supported by the National Institutes of Health Grant (1RO3AI092621-01) and the Center for Excellence in Infection Genomics (CEIG) training grant (DOD #W911NF-11-1-0136). Partial support of this study was from the Jane and Roland Blumberg Professorship in Biology for Dr Arulanandam.

1 Brunham RC, Rey-Ladino J. Immunology of Chlamydia infection: implications for a Chlamydia trachomatis vaccine. Nat Rev Immunol 2005; 5: 149–161.
2 Hafner LM. Pathogenesis of fallopian tube damage caused by Chlamydia trachomatis infections. Contraception 2015; 92: 108–115.
3 Menon S, Timms P, Allan JA, Alexander K, Rombauts L, Horner P et al. Human and pathogen factors associated with Chlamydia trachomatis-related infertility in women. Clin Microbiol Rev 2015; 28: 969–985.
4 Brunham RC, Raguressi R. Chlamydia trachomatis control requires a vaccine. Vaccine 2013; 31: 1892–1897.
5 Mitchell C, Prabhni M. Pelvic inflammatory disease: current concepts in pathogenesis, diagnosis and treatment. Infect Dis Clin N Am 2013; 27: 793–809.
6 Schiborg RV. Chlamydia persistence - a tool to dissect Chlamydia-host interactions. Microbes Infect 2011; 13: 649–662.
7 De Punzi C, Neri E, Metelli P, Bianchi MS, Campa M, Fioretti P. Epidemiology and therapy of Chlamydia trachomatis genital infection in women. J Chemother 1992; 4: 163–166.
8 Murphye C, Murphy AK, Meier PA, Neal Guentzel M, Zhong G, Arulanandam BP. The protective efficacy of chlamydial protease-like activity factor vaccination is dependent on CD4+ T cells. Cell Immunol 2006; 242: 110–117.
9 Murthy AK, Cong Y, Murphy C, Guentzel MN, Forshubeter TG, Zhong G et al. Chlamydia 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: 6722–6729.
10 Cong Y, Jupelli M, Guentzel MN, Zhong G, Murphy 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: 3773–3780.
11 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: 666–676.
12 Li W, Murphy AK, Guentzel MN, Shu J, Forshubeter 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: 3375–3382.
13 Murthy AK, Guentzel MN, Zhong G, Arulanandam BP. Chlamydial protease-like activity factor insights into immunity and vaccine development. J Reprod Immunol 2009; 83: 179–184.
14 Mascellino MT, Boccia P, Olivia A. Immunopathogenesis in Chlamydia trachomatis infected women. ISRN Obstet Gynecol 2011; 2011: 436936.
15 Murphy 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 chlamydia challenge. Vaccine 2011; 29: 2519–2522.
16 Li W, Murphy AK, Lanka GK, Chetty SL, Yu JJ, Chambers JP et al. A T cell epitope-based vaccine protects against chlamydial infection in HLA-DR4 transgenic mice. Vaccine 2013; 31: 5722–5728.
17 Karunakan K, Yu H, Jiang X, Chan Q, Moon KM, Foster LJ et al. Outer membrane proteins preferentially load MHC class II peptides: implications for a Chlamydia trachomatis T cell vaccine. Vaccine 2015; 33: 2159–2166.
18 O'Meara CP, Andrew DW, Beagley KW. The mouse model of Chlamydia genital tract infection: a review of infection, disease, immunity and vaccine development. Cur Med Mol Biol 2013; 14: 396–421.

19 Pan Q, Pais R, Ondjao A, He C, He Q, Omosun Y et al. Comparative evaluation of the protective efficacy of two formulations of a recombinant Chlamydia abortus subunit candidate vaccine in a mouse model. Vaccine 2015; 33: 1865–1872.

20 De Clercq E, Kalmar I, Vanrompay D. Animal models for studying female genital tract infection with Chlamydia trachomatis. Infect Immun 2013; 81: 3060–3067.

21 Ertan G, Wheelhouse N, Wattegedera SR, Longbottom O. New challenges for vaccination to prevent chlamydial abortion in sheep. Comp Immun Microbiol Infect Dis 2012; 35: 271–276.

22 Boje S, Olsen AW, Erenholm K, Agerholm JS, Jungersen G, Andersen P et al. Animal models for genital tract 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: 2272–2282.

23 Jacob E, Drews M, Stuhlert A,Buttnier C, Klein PJ, Kist M et al. Immunological reaction of guinea-pigs following intranasal Mycoplasma pneumoniae infection and immunization with the 16 kDa adherence protein. J Gen Microbiol 1988; 134: 473–479.

24 Hogarth PJ, Jahans KJ, Hecker R, Hewinson RG, Chambers MA. Evaluation of adjuvants on animal health. FEMS Immunol Med Microbiol 2013; 73: 1868–1872.

25 Jacobs ES, Reddell RJ, Buttnier C, Klein PJ, Kist M et al. Immunological reaction of guinea-pigs following intranasal Mycoplasma pneumoniae infection and immunization with the 16 kDa adherence protein. J Gen Microbiol 1988; 134: 473–479.

26 O'Meara CP, Armitage CW, Harvie MC, Andrew DW, Timms P, Lycke NY et al. Animal models for the study of Chlamydia Psittaci infection using microplates arrayed with 156 chlamydial fusion proteins. Infect Immun 2006; 74: 1490–1499.

27 Coler RN, Bhattachar J, Bhattachar J, Bhatia A, Maisonneuve JF, Probst P, Barth B, Ovendale P et al. Genetic transformation of a clinical (genital tract), plasmid-free isolate of Chlamydia trachomatis - engineering the plasmid as a cloning vector. PLoS ONE 2013; 8: e59195.

28 Li LX, McSorley SJ. A re-evaluation of the role of B cells in protective immunity to Chlamydia pneumoniae. Immunol Lett 2015; 164: 88–93.

29 Rank RG. Animal models for urogenital infections. Methods Enzymol 1994; 235: 83–93.

30 Andrew DW, Hafner LM, Beagley KW, Timms P. Partial protection against chlamydioidal reproductive tract infection by a recombinant major outer membrane protein CpG/cholera toxin intranasal vaccine in the guinea pig. Chlamydia psittaci model. J Reprod Immunol 2011; 91: 9–16.

31 Batteiger BE, Rank RG, Bavoil PM, Soderberg LS. Partial protection against genital reinfection by immunization of guinea-pigs with isolated outer-membrane proteins of the chlamydial agent of guinea-pig inclusion conjunctivitis. J Gen Microbiol 1993; 139: 2965–2972.

32 Volp K, Mathews S, Timms P, Hafner L. Peptide immunization of guinea pigs against Chlamydia psittaci (GPIC agent) infection induces good vaginal secretion antibody response, in vitro neutralization and partial protection against live challenge. Immunol Cell Biol 2001; 79: 245–250.

33 Wali S, Gupta R, Veselenak RL, Li Y, Yu JJ, Murthy AK et al. Use of a pig vaccine specific transcriptome array for evaluation of protective immunity against genital chlamydial infection following intranasal vaccination in guinea pigs. PLoS ONE 2014; 9: e114261.

34 Cotter TW, Ramsey KH, Manirpani GS, Poulsen CE, Byrne GI. Dissemination of Chlamydia trachomatis chronic genital tract infection in gamma interferon gene knockout mice. Infect Immun 1997; 65: 2145–2152.

35 Johansson M, Schon K, Ward M, Lycke N. Genital tract infection with Chlamydia trachomatis fails to induce protective immunity in gamma interferon receptor-deficient mice despite a strong local immunoglobulin A response. Infect Immun 1997; 65: 1032–1044.

36 de Jonge MI, Keizer SA, El Moussaoui HM, van Dorsten L, Azzawi R, van Zuelenk HM et al. A novel guinea pig model of Chlamydia psittaci genital tract infection. Vaccine 2011; 29: 5994–6001.

37 Dong F, Zhong Y, Arulanandam B, Zhong G. Production of a proteolytically active protein, chlamydial protease/protease-like activity factor, by five different Chlamydia species. Infect Immun 2005; 73: 1807–1813.

38 O'Meara CP, Armitage CW, Harvie MC, Andrew DW, Timms P, Lycke NY et al. Animal models for the study of Chlamydia Psittaci infection using microplates arrayed with 156 chlamydial fusion proteins. Infect Immun 2006; 74: 258–270.

39 Rank RG, Barron AL. Effect of estradiol on anti-chlamydial immunity in guinea pigs after repeat vaginal inoculation with the chlamydial agent of guinea pig inclusion conjunctivitis. Sex Transm Dis 1995; 22: 48–54.

40 Rank RG, White HJ, Hough AJ Jr, Pasley JN, Barron AL. Effect of estradiol on anti-chlamydial immunity in guinea pigs after repeat vaginal inoculation with the chlamydial agent of guinea pig inclusion conjunctivitis. Sex Transm Dis 1995; 22: 48–54.

41 Li W, Murthy AK, Guentzel MN, Chambers JP, Forshhuber 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: 3942–3949.

42 Murthy AK, Chaganti BK, Li W, Guentzel MN, Chambers JP, Seshu J et al. A limited role for antibody in protective immunity induced by CpAF and CpG vaccination against primary genital Chlamydia muridarum infection. FEMS Immunol Med Microbiol 2009; 55: 271–279.

43 Tifrea DP, Pal S, Popol JL, Cocco MJ, de la Maza LM. Increased immunoreactivity of MMP7 epitopes in a vaccine formulated with amphipols may account for the very robust protection elicited against a vaginal challenge with Chlamydia muridarum. J Immunol 2014; 192: 5201–5213.

44 Rank RG, White HJ, Hough AJ Jr, Pasley JN, Barron AL. Effect of estradiol on anti-chlamydial genital infection of female guinea pigs. Infect Immun 1982; 38: 699–705.

45 Silva EG, Tornos C, Deavers M, Kaisman K, Gray K, Gershenson D. Induction of epithelial neoplasms in the ovaries of guinea pigs by estrogenic stimulation. Gynecol Oncol 1996; 61: 240–246.

46 Rank RG, Sanders MM, Patton DL. Increased incidence of oviduct pathology in the guinea pig after repeat vaginal inoculation with the chlamydial agent of guinea pig inclusion conjunctivitis. Sex Transm Dis 1995; 22: 48–54.

47 Rank RG, White HJ, Hough AJ Jr, Pasley JN, Barron AL. Effect of estradiol on anti-chlamydial immunity in guinea pigs after repeat vaginal inoculation with the chlamydial agent of guinea pig inclusion conjunctivitis. Sex Transm Dis 1995; 22: 48–54.

48 Rank RG, White HJ, Hough AJ Jr, Pasley JN, Barron AL. Effect of estradiol on anti-chlamydial immunity in guinea pigs after repeat vaginal inoculation with the chlamydial agent of guinea pig inclusion conjunctivitis. Sex Transm Dis 1995; 22: 48–54.