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Comparison of immune responses and protective efficacy of intranasal prime-boost immunization regimens using adenovirus-based and CpG/HH2 adjuvanted-subunit vaccines against genital *Chlamydia muridarum* infection

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ABSTRACT

An efficacious Chlamydia vaccine is urgently needed to control *Chlamydia* infections. Heterologous prime-boost vaccination regimens are emerging as a promising strategy for preventing intracellular viral and bacterial infections. However, it remains to be determined if this regimen would be a feasible and effective approach for *Chlamydia* infection. In this study, we examined the immune response and the protective efficacy induced by various vaccination regimens using a recombinant adenovirus vector expressing the *Chlamydia* antigen CPAF (AdCPAF) and recombinant CPAF (rCPAF) subunit vaccines formulated with CpG oligodeoxynucleotides and/or a synthetic immunomodulatory peptide HH2 as adjuvants. A single dose of AdCPAF stimulated potent antibody production but weak cellular immune responses in mice. A booster rCPAF vaccine formulated with both CpG and HH2, but not CpG alone or HH2 alone, showed robust adjuvant effects on induction of Th1-biased cellular immune responses in mice primed with AdCPAF. In contrast, a homologous regimen using rCPAF/CpG/HH2 subunit vaccine for both priming and boosting induced a weak antibody response, but potent cellular immunity with a mixed Th1/Th17 profile. Despite the disparities observed in humoral and cellular immune responses, both the heterologous and homologous prime-boost regimens conferred significant immune protection against genital *Chlamydia muridarum* challenge in C3H/HeN and BALB/c mice.

1. Introduction

Urogenital infections caused by *Chlamydia trachomatis* are the most commonly reported bacterial sexually transmitted infection (STI) in humans worldwide. Although antibiotics can effectively control *Chlamydia* infections, many infected individuals do not seek medical treatment due to lack of overt clinical symptoms. Left untreated, *Chlamydia* infection in the lower genital tract can potentially ascend to the upper genital tract and lead to pelvic inflammatory diseases, ectopic pregnancy and tubal factor infertility [1,2]. There is an urgent need for developing a safe and effective vaccine for controlling *Chlamydia* infection and preventing *Chlamydia*-caused diseases.

Both animal and human studies have established a vital role of T-cell-mediated immunity, predominantly CD4+ T cells, and a complementary role of humoral immunity in host resistance to *Chlamydia* infection [1,3]. Th1 immunity, defined by production of IFN-γ, plays a vital role in mediating anti-*Chlamydia* immunity [1]. Mice deficient in IFN-γ or IFN-γ receptors were unable to clear *Chlamydia muridarum* infection [4,5]. Adoptive transfer of CD4+ T cells that were activated by vaccination significantly enhanced host resistance to subsequent *C. muridarum* challenge in IFN-γ-receptor-competent, but not IFN-γ-receptor-deficient mice [6,7]. These studies suggest that the IFN-γ/Th1 immune response is a good surrogate marker for predicting the efficacy of *Chlamydia* vaccines. Recently, the IL-17/Th17 response

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was also reported as an integral part of the cellular immune response to *Chlamydia* infection in both mice and humans [8,9]. Administration of anti-IL-17-neutralizing-antibody in mice results in attenuation in *C. muridarum* clearance, suggesting a protective role of IL-17/Th17 response in anti-*Chlamydia* immunity [10,11]. However, *Chlamydia*-susceptible C3H/HeN and BALB/c mice tend to develop heightened IL-17/Th17 responses relative to *Chlamydia*-resistant C57BL/6 mice during primary *Chlamydia* infection [8,12], suggesting that excess IL-17/Th17 response may be detrimental to host resistance. Thus, how IL-17/Th17 response correlates with vaccination-induced host resistance remains unclear.

Recombinant adenoviral-based (rAd-based) vaccines have emerged as a vaccine platform to target many human infectious diseases including HIV, tuberculosis (TB), malaria and influenza [13–16]. Due to a natural tropism to respiratory mucosal epithelial cells, rAd-based vaccines are particularly attractive for developing needle-free nasal vaccines against mucosal pathogens. For instance, intranasal (i.n.) immunization with rAd-based vaccine activates both CD4+ and CD8+ T cells and results in distribution of activated-T cells in the airway lumen and potent immune protection against respiratory infections caused by *Mycobacterium tuberculosis* [17,18], influenza virus [19,20] and respiratory syncytial virus [21]. In immunization with rAd-based vaccine is also able to stimulate humoral and cellular immunity at distal genital tract sites and protect against herpes simplex virus [22,23]. Yet, the development of rAd-based nasal vaccine against genital *Chlamydia* infection has not been reported. While the immune responses induced by viral-based vaccines are demonstrated to be predominantly Th1-type [16,24], i.n. immunization with soluble antigen preferentially stimulates Th17 response regardless of the adjuvant used [25]. Thus, it is reasonable to hypothesize that a vaccination regimen using i.n. delivery of a viral-based vaccine and a subunit vaccine might be able to stimulate both Th1 and Th17 immune responses that are required for host resistance against *Chlamydia*. Since anti-vector immunity may have an impeding effect on the potency of rAd-based vaccines, a more desirable vaccination regimen in practice may need to use a rAd-based vaccine for priming before the general population acquires neutralization antibody around age of 3–5 and to use a subunit vaccine for boosting subsequrently [24,26]. However, it is unclear whether immune responses induced by rAd-based vaccines can be easily boosted by any subunit vaccine, or, it requires specific adjuvants for formulating subunit boosting vaccines.

CpG motif, the agonist of Toll-like receptor (TLR) 9, is well-known to stimulate a Th1 immune response and has been tested as a vaccine adjuvant. The adjuvant effects of CpG are further enhanced by cationic host defensin peptides (HDP) and their synthetic analogs named innate defence regulator peptides (IDR) [27–31]. HH2 is a synthetic IDR optimized for its immunomodulatory activities, which can complex with CpG via strong electrostatic interactions and stimulate potent antibody production and B cell expansion [27,28]. However, the effect of CpG/HH2 complex relative to CpG alone or HH2 alone at regulating cellular immune responses such as Th1/Th17 immune profile is unclear.

In this study, we developed several vaccine formulations based on *Chlamydia* Protease (or Proteosome-like) Activity Factor (CPAF) antigen, which is a highly conserved serine protease in all *Chlamydia* species [32], and only expressed by the replicating form of reticulate body following infection [33,34]. A rAd-based vaccine expressing CPAF (AdCPAF) and recombinant CPAF (rCPAF) subunit vaccines formulated with CpG (rCPAF/CpG) or HH2 peptide (rCPAF/HH2) or CpG/HH2 complex (rCPAF/CpG/HH2) were used in either heterologous or homologous prime-boost regimens. The objectives of this study were (1) to identify a subunit vaccine that can boost immune responses induced by AdCPAF; (2) to compare immune profile, particularly Th1/Th17 profile, induced by the heterologous prime-boost regimen using AdCPAF-priming and subunit vaccine-boosting and the homologous prime-boost regimen using subunit vaccine for both priming and boosting; and (3) to define protective efficacy of different vaccination regimens in an animal model of genital *C. muridarum* infection.

2. Materials and methods

2.1. Mice

Six- to 10-week-old male or female C3H/HeN, BALB/c and C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). Mice were housed at the Izaak Walton Killam Health Centre (IWK) animal facility under pathogen-free conditions. All animal procedures were approved by the Ethics Committee according to the Canadian Council for Animal Care guidelines.

2.2. Construction of recombinant AdCPAF

The CPAF DNA fragment was amplified by PCR and sub-cloned into the shuttle plasmid vector pML01. This plasmid (pJW47) was used to co-transfect 293 cells with a rescuing vector pBHGloxΔE1,3Cre (Fig. 1a). The site-specific recombination was catalyzed by recombinase Cre expressed by the rescue vector [35]. Successful recombination led to visible viral plaques in 293 cells, which were screened for correct CPAF insertion in the viral genome. The viral plaque that carried the CPAF transgene insert was confirmed for proper CPAF-transgene expression by RT-PCR (Fig. 1b) and saved as an AdCPAF seed-stock for all future experiments. The presence of secreted CPAF was confirmed in AdCPAF-infected A549 culture supernatant by a CPAF-specific monoclonal antibody (clone 54b) (Fig. 1c) [32]. The empty adenoviral vector Addl70-3 was used as negative controls (Ctrl Ad) in all experiments. AdCPAF and Addl70-3 were amplified, purified and titrated according to the protocols previously described [18,36].

2.3. Construction and production of recombinant His-tagged CPAF protein (rCPAF)

The CPAF DNA was also sub-cloned into the pET-16b expression vector that carries an N-terminus His-Tag (Novagen, Madison, WI). The recombinant His-Tagged-CPAF protein was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) in *Escherichia coli* BL21 (DE3) strain and purified through HIS-Select® Cobalt Affinity column (Sigma–Aldrich, Oakville, Ontario) under denaturing conditions. The purity of rCPAF was confirmed by western blot probed with anti-CPAF monoclonal antibody 54b [32]. The protein concentration was determined using Pierce BCA Protein Assay Reagent Kit (Thermo Fisher, Waltham, MA) and endotoxin levels were measured using *Limulus* Amebocyte Lysate (LAL) test (Associates of Cape Cod, East Falmouth, MA). rCPAF protein used in the study had an endotoxin content of less than 1 EU/μg.

2.4. Intranasal (i.n.) immunization with AdCPAF and adjuvanted-CPAF subunit vaccines

All immunizations were carried out via i.n. delivery as previously described [18,36–39]. Adenoviral vectors (AdCPAF or Ctrl Ad) were diluted to 5×10^8 plaque-forming units (PFU) per 25 μL in sterile PBS. CPAF subunit vaccines were formulated by mixing 15 μg of rCPAF with 10 μg of CpG (Type C, TCGTCGTTTTCGGCGCG CGCCG) and/or 50 μg of HH2 peptide (VLQRIRVAVIRA) in a total of 25 μL of sterile PBS. For mice receiving prime-boost immunization regimens, boosters were given 2 weeks after priming. Mock-immunized mice received 25 μL of sterile PBS and served as

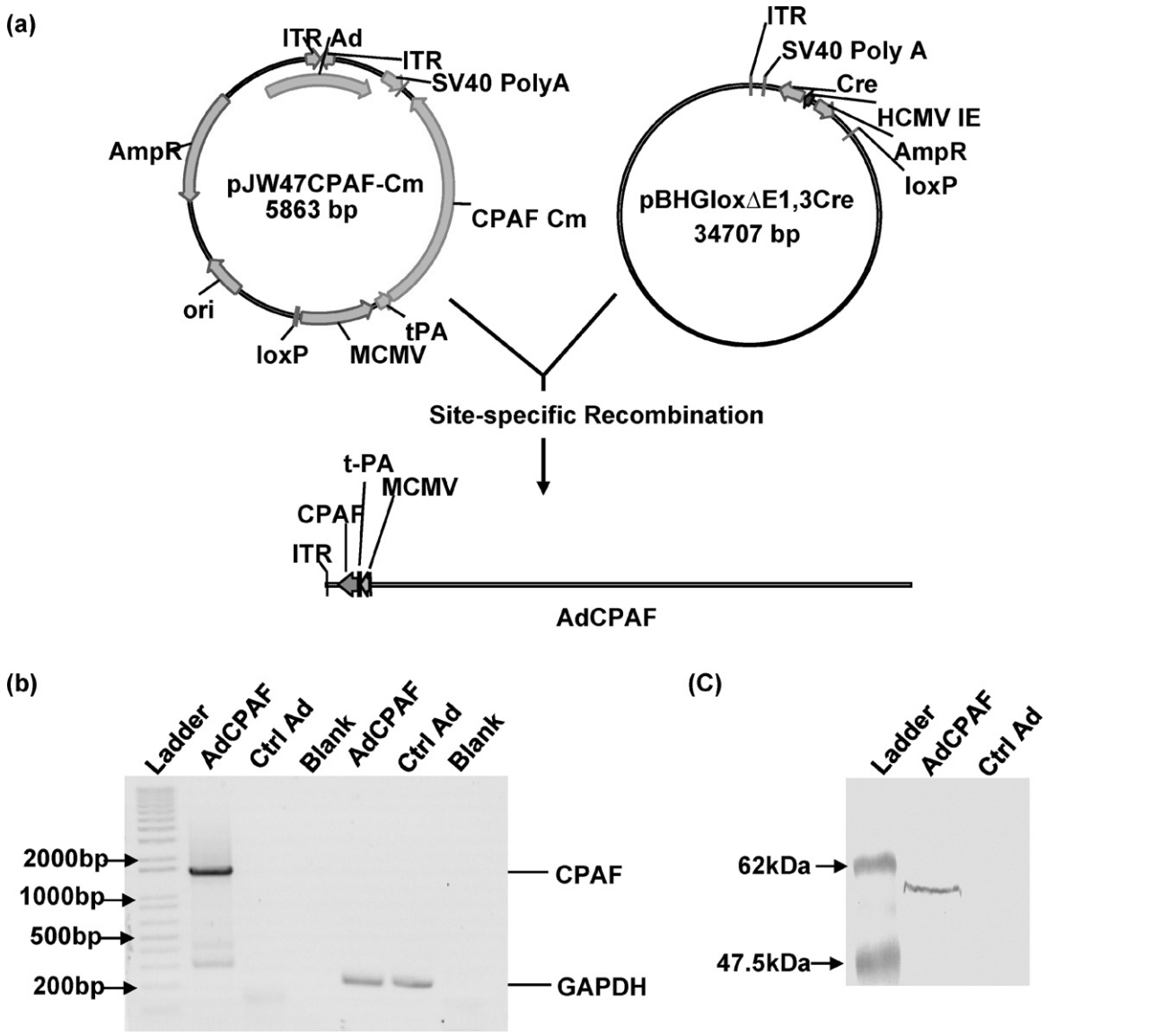

Fig. 1. Construction and characterization of AdCPAF *in vitro*. (a) A shuttle plasmid vector pJW47CPAF-Cm was constructed by multiple cloning steps and used to co-transfect 293 cells with rescuing vector pBHGloxΔE1,3Cre. The AdCPAF was made after site-specific recombination catalyzed by Cre recombinase. (b) Detection of CPAF transcript by RT-PCR in 293 cells 24 h post AdCPAF or Ctrl Ad infection. GAPDH was used as an internal control for RNA input. (c) Detection of secreted CPAF by western blotting in A549 cell culture supernatants 48 h post AdCPAF or Ctrl Ad infection.

negative controls. Positive control mice received live *C. muridarum* infection as described below.

2.5. Detection of CPAF-specific antibody titer in sera and genital swabs

At 4-weeks after the last immunization, sera were collected from mice receiving various vaccination regimens by cardiac puncture, and vaginal swabs were taken using calcium alginate fibber tipped-swabs and placed in 300 μL of sterile sucrose–phosphate–glutamic acid (SPG) buffer. After 30 min of agitation in SPG buffer at 4 °C, swabs were removed and the supernatants were stored at −20 °C. For measuring antibody titer in sera and genital swabs, NUNC Maxisorp™ plates (Thermo Fisher, Waltham, MA) were coated with 5 μg/well of rCPAF in 0.05 M sodium bicarbonate pH 9.6 overnight at 4 °C. Plates were washed, blocked and then loaded with the genital swab samples or serum samples. Following overnight incubation at 4 °C, unbound samples were washed away and plates were probed with alkaline phosphatase (AP)-conjugated anti-mouse-whole Ig secondary antibody (Sigma–Aldrich, Oakville, Ontario), developed and read at OD_{405} nm using a BioTek synergy HT plate reader (BioTek, Winooski, VT). The CPAF-specific antibody titer was calculated as the reciprocal dilution at which the OD_{405} nm was 0.050 above background. Similarly, the titers of IgG1, IgG2a and IgA were determined by using AP-conjugated anti-mouse IgG1 (Southern Biotech, Birmingham, AL), AP-conjugated anti-mouse IgG2a (Southern Biotech, Birmingham, AL) and AP-conjugated-anti-mouse IgA (Sigma–Aldrich, Oakville, Ontario) secondary antibodies, respectively.

2.6. Ex vivo antigen re-stimulation assay and intracellular cytokine staining (ICCS)

At various time points post-vaccination, lungs, spleens and iliac lymph nodes (ILNs) were removed from mice (3–4 mice per group). Single cell suspensions were isolated and seeded into 96-well plates (0.5×10^6 per well) in RPMI 1640 medium containing

10% FBS, 50 U/mL penicillin, 50 g/mL streptomycin, and 2 mmol/L of L-glutamine and re-stimulated with purified rCPAF at 5 μg/mL, or heat-killed, purified *C. muridarum* elementary body antigen (Heat-CmAg), or UV-inactivated AdAg (UV-AdAg), or medium alone for 72 h at 37 °C. The culture supernatants were used for detecting cytokine content by ELISA. For examining the frequency of CPAF-specific, IFN-γ-producing T-cell, cells were re-stimulated in U-bottom 96-well plates with or without rCPAF for 48 h, and then treated with brefeldin A at a final concentration of 3 μg/mL for an additional 5–6 h at 37 °C. The cells were harvested and ICCS was conducted by using Cytofix/Cytoperm kits (BD Pharmingen) as described previously [8,18]. Briefly, the cells were first surface stained with FITC-CD4, PE-CD8α and PerCP-Cy5.5-CD3e, then fixed, permeabilized and intracellularly labeled with Alexa-647-IFN-γ or Alexa-647-Rat IgG1 (all antibodies were purchased from eBioscience, San Diego, CA). A total of 100,000–300,000 events were collected on a FACScalibur (BD Biosciences, Sunnyvale, CA), and the data were analyzed using WinMDI2.8 software (Scripps Institute, La Jolla, CA).

2.7. Respiratory and genital infection with Chlamydia

The mouse pneumonitis biovar, *C. muridarum*, was propagated in McCoy cells (ATCC, Manassas, VA) according to procedures described previously [8]. The infectious elementary bodies were purified by discontinuous density gradient centrifugation using 30% Isovue-370 (Bracco Diagnostics, Princeton, NJ) and 50% sucrose (Sigma, Oakville, Ontario). For setting up pre-infected mice, a dose of 1×10^3 inclusion-forming unit (IFU) *C. muridarum* was diluted in 25 μL of SPG buffer and delivered i.n. into isoflurane-anesthetized-mice as previously described [8]. To examine the efficacy of various vaccination regimens, mice were challenged with live *C. muridarum* (5×10^4 IFU) in the genital tract 4 weeks after the booster immunization. Ten and three days prior to *C. muridarum* challenge, mice were injected subcutaneously with 2.5 mg of Depo-provera (Pfizer Canada, Kirkland, QC) to synchronize the estrous cycle. Every six days following infection, vaginal swabs were taken and stored at −80 °C for bacterial load quantification by IFU assay.

2.8. Quantification of bacterial load in Chlamydia-challenged mice

To determine the bacterial load in *Chlamydia*-challenged mice, McCoy cells were grown in Minimum Essential Medium (MEM) plus 5% FBS, 2 μg/mL fungizone and 25 μg/mL of gentamicin in 96-well plates and then inoculated with genital swab samples and serial dilutions of purified-*Chlamydia* with known titers as standards [8]. Infected-McCoy cells were incubated with Growth Media (MEM plus 10% FBS, 0.5% glucose, 0.225% sodium bicarbonate, 20 mM HEPES, 2% L-glutamine, 2% vitamins, 10 μg/mL gentamicin and 1 μg/mL cyclohexamide) at 37 °C and 5% CO_2. After 48 h of incubation, the cell monolayer was fixed with 100% methanol, and stained with a genus-specific biotin-conjugated rabbit anti-*Chlamydia* polyclonal antibody (Biodesign International, Saco, ME) and a streptavidin-conjugated Texas red (Sigma–Aldrich, St. Louis, MO) secondary antibody. The fluorescent inclusions were counted (10 fields per well) using an automatic stage Olympus IX71 microscope (Olympus, Tokyo, Japan) and the number of inclusion forming units was calculated accordingly.

2.9. Cytokine measurement and histology

The level of cytokines in the supernatants was measured by using mouse-specific ELISA kits (R&D Systems, Minneapolis, MN). The sensitivity of detection for IFN-γ, IL-17A, IL-17F, IL-4, and IL-10 was 2–5 pg/mL.

3. Results

3.1. Characterization of adenoviral vector expressing CPAF (AdCPAF) in vitro

Recombinant adenoviral vectors expressing a secreted form of antigen stimulate potent antigen-specific CD4+ and CD8+ T-cell responses *in vivo* [18,40]. Thus, AdCPAF was designed to encode a secreted form of whole length CPAF by replacing its endogenous signal peptide with human t-PA signal peptide sequence (Fig. 1a). A strong gene-product with the predicted size of the CPAF gene was detected by RT-PCR in the total RNA isolated from A549 cells that were infected with AdCPAF but not with the Ctrl Ad; whereas comparable amounts of house-keeping gene GAPDH were present in both AdCPAF-infected and Ctrl Ad-infected samples (Fig. 1b). Furthermore, we were able to detect CPAF protein in the culture supernatants derived from A549 cells infected with AdCPAF but not Ctrl Ad by western blotting using the CPAF-specific monoclonal antibody 54b as the probe (Fig. 1c), indicating that AdCPAF is able to produce soluble CPAF by transduced mammalian cells.

3.2. AdCPAF immunization stimulates antigen-specific immune responses in vivo

After confirming the correctness of AdCPAF *in vitro*, we first examined whether AdCPAF was able to stimulate CPAF-specific immune responses *in vivo*. C57BL/6 mice were immunized intramuscularly with AdCPAF or Ctrl Ad. Some mice were given PBS or live *C. muridarum* in the airway as controls. Splenocytes were isolated 10 days post-immunization and re-stimulated with rCPAF, Heat-CmAg and UV-AdAg *ex vivo*. The contents of IFN-γ, IL-4 and IL-17, representing induction of Th1, Th2 and Th17 immune responses, respectively, in the culture supernatants were measured by ELISA. As shown in Fig. 2a and b, rCPAF stimulation triggered robust IFN-γ and modest IL-17 production in the splenocytes derived from mice immunized with AdCPAF, but not with Ctrl Ad ($p < 0.001$). Such CPAF-specific immune responses were also markedly induced in mice receiving live *C. muridarum* infection, but not PBS injection ($p < 0.001$). UV-AdAg stimulation resulted in comparable levels of IFN-γ production in AdCPAF- and Ctrl Ad-immunized mice. In contrast, these adenoviral antigen-specific cytokine responses were not detectable in live *C. muridarum*-infected mice or mock-immunized mice, confirming the antigen specificity of cytokine production measured in this assay. Consistent with the understanding that CPAF was only expressed by replicating form of *Chlamydia* [33], stimulation with Heat-CmAg did not trigger significant cytokine responses in AdCPAF-immunized mice. However, robust IFN-γ and IL-17 productions were observed in live *C. muridarum* infected mice upon Heat-CmAg stimulation. We could not detect significant IL-4 production in the culture supernatants under any stimulation conditions.

To understand whether AdCPAF is immunogenic in mice with diverse genetic backgrounds, an additional experiment was carried out in C57BL/6, BALB/c and C3H/HeN mice. We found that AdCPAF was able to stimulate CPAF-specific immune responses in all mouse strains examined. As shown in Fig. 2c, AdCPAF immunization triggered both CD4+ and CD8+ T-cell activation as indicated by intracellular IFN-γ-staining in the splenocytes upon rCPAF stimulation, suggesting that immunization with AdCPAF is able to stimulate CPAF-specific immune responses in different mouse strains *in vivo*.

Table 1
Vaccination regimens using AdCPAF and rCPAF subunit vaccines.

Group	Priming (day 0)	Boosting (day 15)	Designation
1	AdCPAF	No boost	Single dose
2	AdCPAF	rCPAF	
3	AdCPAF	rCPAF/CpG	
4	AdCPAF	rCPAF/HH2	
5	AdCPAF	rCPAF/CpG/HH2	Heterologous prime-boost
6	rCPAF/CpG/HH2	rCPAF/CpG/HH2	Homologous prime-boost
7	Control Ad	No boost	
8	Control Ad	rCPAF	
9	Control Ad	rCPAF/CpG	
10	Control Ad	rCPAF/HH2	
11	Control Ad	rCPAF/CpG/HH2	
12	PBS	PBS	Mock

3.3. Different vaccination regimens using AdCPAF and rCPAF-based subunit vaccines stimulate distinct humoral immune responses in vivo

After characterizing AdCPAF *in vitro* and *in vivo*, we set to identify a mucosal vaccination regimen using AdCPAF and CPAF subunit vaccines formulated with different adjuvants. To this end, C3H/HEN mice were i.n. immunized according to different vaccination regimens (see Table 1). Mice were euthanized 4 weeks after the booster immunization and the immune responses to vaccination were assessed. All of the mice primed with AdCPAF (with or without a booster) developed CPAF-specific antibody titers ranging from 10^4 to 10^5 in the sera (Fig. 3a). In contrast, mice primed with Ctrl Ad had significantly lower levels of CPAF-specific antibody compared to their AdCPAF-primed counterparts ($p < 0.001$). Boosting with rCPAF/CpG, or rCPAF/HH2, or rCPAF/CpG/HH2 did not appear to greatly improve serum antibody responses compared to a single dose of AdCPAF immunization (Fig. 3a). However, mice receiving a single dose of AdCPAF or the heterologous vaccination regimen with rCPAF/CpG/HH2 boosting generated CPAF-specific antibody titers that were significantly higher than those receiving the homologous prime-boost regimen (Fig. 3b). We also examined IgG_{2a} and IgG_1 as an indirect measure of Th1/Th2 immune profile. Again, AdCPAF, but not Ctrl Ad, immunization triggered high titers of CPAF-specific IgG_{2a} and IgG_1 antibodies in general (Fig. 3c, e and g). However, mice that received a single dose of AdCPAF or the heterologous prime-boost immunization regimens with CpG or CpG/HH2 complex as adjuvants developed IgG_{2a} to IgG_1 titer ratios of greater than one in all mice tested, indicating a biased Th1 immune profile (Fig. 3c, e and g). In contrast, mice primed with AdCPAF and boosted with rCPAF alone stimulated IgG_{2a} to IgG_1 titer ratios of less than one in all mice, highlighting CPAF alone has an intrinsic property to promote a Th2-biased immune response (Fig. 3c, e and g). The homologous prime-boost regimen was found to generate CPAF-specific antibodies with an IgG_{2a} to IgG_1 titer ratio of approximately one (Fig. 3d, f and g), indicating a mixed Th1/Th2 type response. IgG_{2a} to IgG_1 titer ratios could not be calculated for Ctrl Ad or mock-immunized mice as very little CPAF-specific antibody could be detected in any samples. The overall concentration of CPAF-specific antibody in the genital tract was low, requiring undiluted samples for detection. However, based on the OD reading, a significant induction of CPAF-specific antibody was observed in mice immunized with AdCPAF but not Ctrl Ad (Fig. 3h). Similar to what we observed in the serum, the heterologous prime-boost regimen did not improve the level of CPAF-specific antibodies in the genital tract compared to a single dose of AdCPAF (Fig. 3h). CPAF-specific IgA was also detectable in the female genital tract upon immunization with AdCPAF, which, again, was not improved by boosting with rCPAF/CpG/HH2 (Fig. 3i).

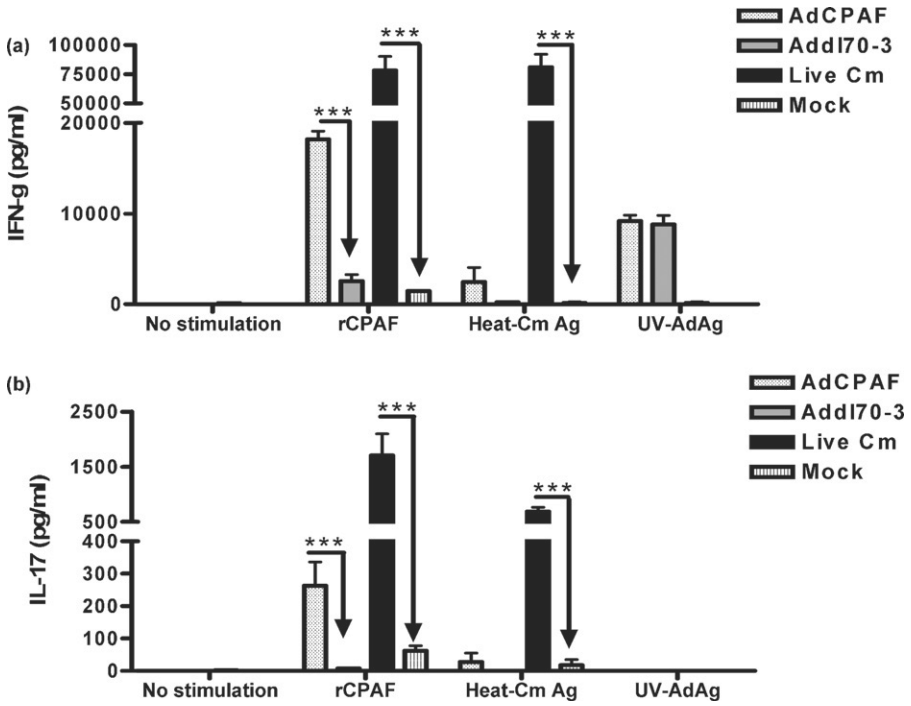

Fig. 2. Characterization of immunogenicity of AdCPAF *in vivo*. (a and b) Splenocytes were isolated from C57BL/6 mice 10 days after receiving AdCPAF or Ctrl Ad vector or live *C muridarum* or PBS and stimulated with various antigens as indicated for 72 h. IFN-γ and IL-17 content in the culture supernatants were measured by ELISA. The data are presented as the mean ± SEM ($n = 3$–4 per group). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ using two-way ANOVA test. (c) ICCS in splenocytes isolated from C57BL/6, C3H/HeN and BALB/c mice 10 days after AdCPAF immunization. The percent of IFN-γ-producing CD4+ or CD8+ T cells with or without CPAF stimulation in each strain is indicated in each panel. Rat IgG1 was used as the isotype control for anti-IFN-γ.

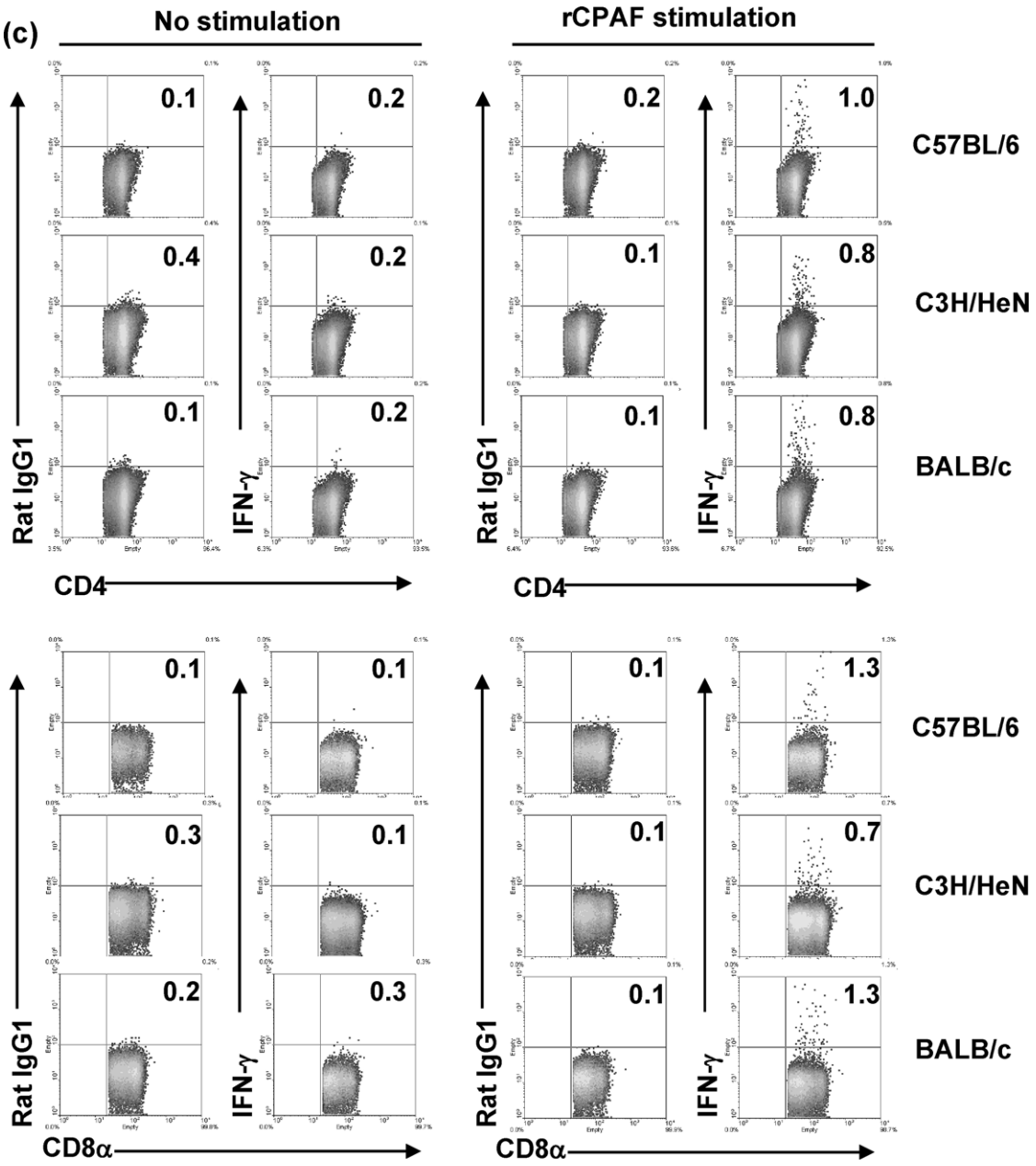

Fig. 2. (*continued*).

3.4. Different vaccination regimens using AdCPAF and rCPAF-based subunit vaccines stimulate distinct profiles and distribution of cellular immune responses in vivo

The CPAF-specific cellular responses were also assessed 4 weeks after various i.n. immunization regimens using single cell suspensions derived from lung tissue. Unlike antibody responses, a single dose of AdCPAF immunization was relatively ineffective at stimulating CPAF-specific cellular responses. Nevertheless, mice receiving the heterologous prime-boost regimen with AdCPAF-priming and rCPAF/CpG/HH2-boosting displayed robust CPAF-specific IFN-γ and IL-17 recall responses (Fig. 4a and b), but little or no IL-10 and IL-4 recall responses (data not shown). In contrast, boosting with rCPAF/CpG, or rCPAF/HH2, or rCPAF alone only had marginal impacts on the level of IFN-γ and IL-17 (Fig. 4a and b), indicating an additive adjuvant effect between the CpG and HH2 on cellular immune responses. Despite low antibody responses, the homologous prime-boost regimen stimulated very strong cellular responses of a mixed Th1/Th17 immune profile (Fig. 4a and b). We noticed a significant difference in the ratio of IFN-γ/IL-17 responses between the heterologous and the homologous prim-boost regimens (Fig. 4c).

Subsequently, we examined how different immunization regimens (single AdCPAF, heterologous and homologous prime-boost using CpG/HH2 complex as adjuvant) affect on CPAF-specific cellular immune responses in distal organs such as spleen and genital tract. Of interest, while the CPAF-specific immune profile in terms of IFN-γ and IL-17 production induced by different immunization regimens was also observed in the spleen (Fig. 4d), very little immune responses were detected in the ILN draining the genital tract following AdCPAF or the heterologous prime-boost immunization (Fig. 4e). In contrast, mice received the homologous

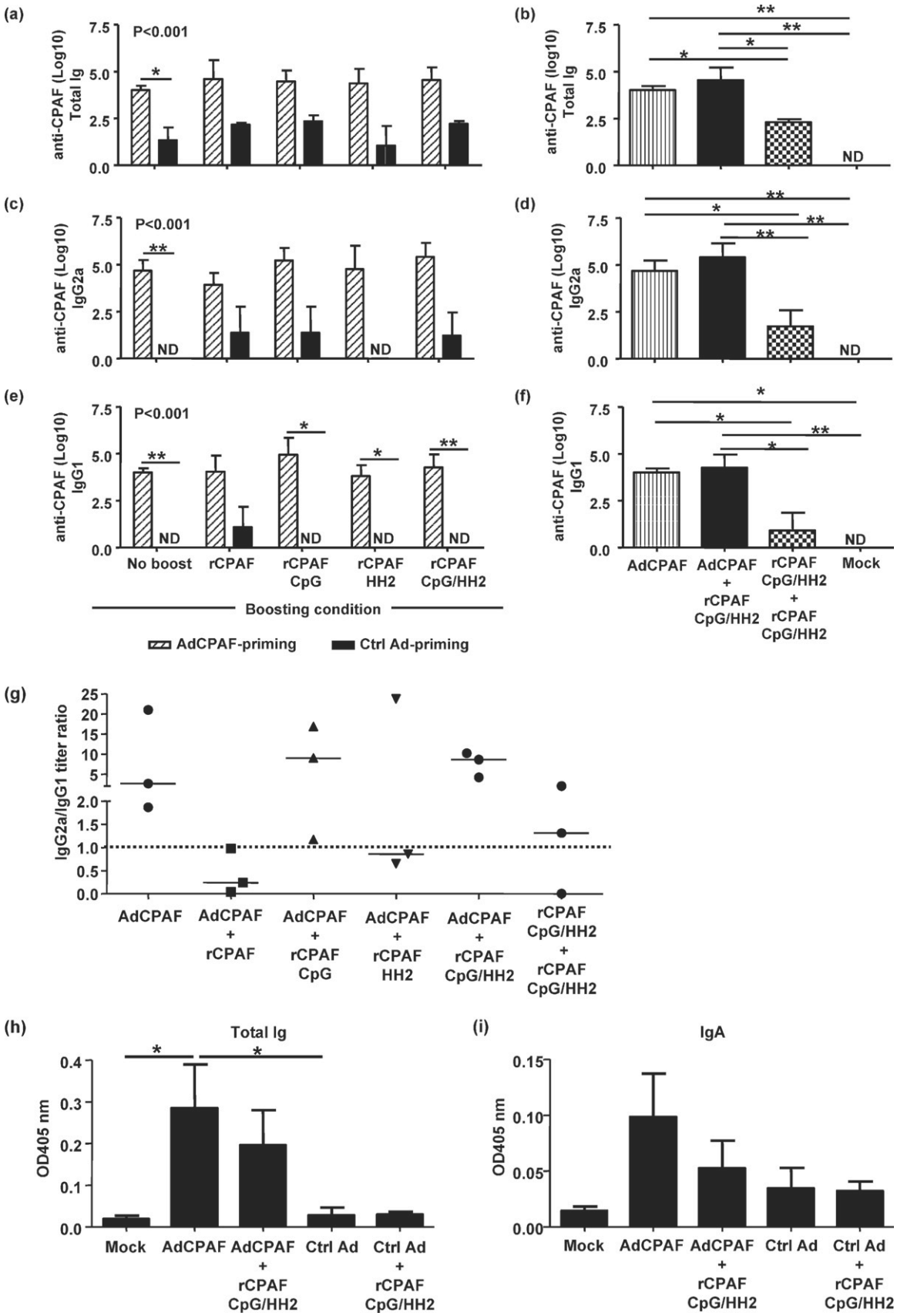

Fig. 3. Antibody production triggered by different vaccination regimens. C3H/HeN mice were immunized i.n. with various vaccination regimens as indicated and sacrificed four weeks after the booster immunization. (a–f) Serum samples were collected and tested for presence of CPAF-specific total antibody (a, b), IgG_{2a} (c, d) and IgG_1 (e, f) using an indirect ELISA assay. Results are presented as mean + SEM ($n = 3$ per group). A two-way ANOVA test followed by Bonferroni post-test was used to compare AdCPAF-primed and Ctrl Ad-primed vaccination regimens in panels a, c and e. A one-way ANOVA followed by a Post-Hoc Tukey's multiple comparison test was used for panels b, d and f.

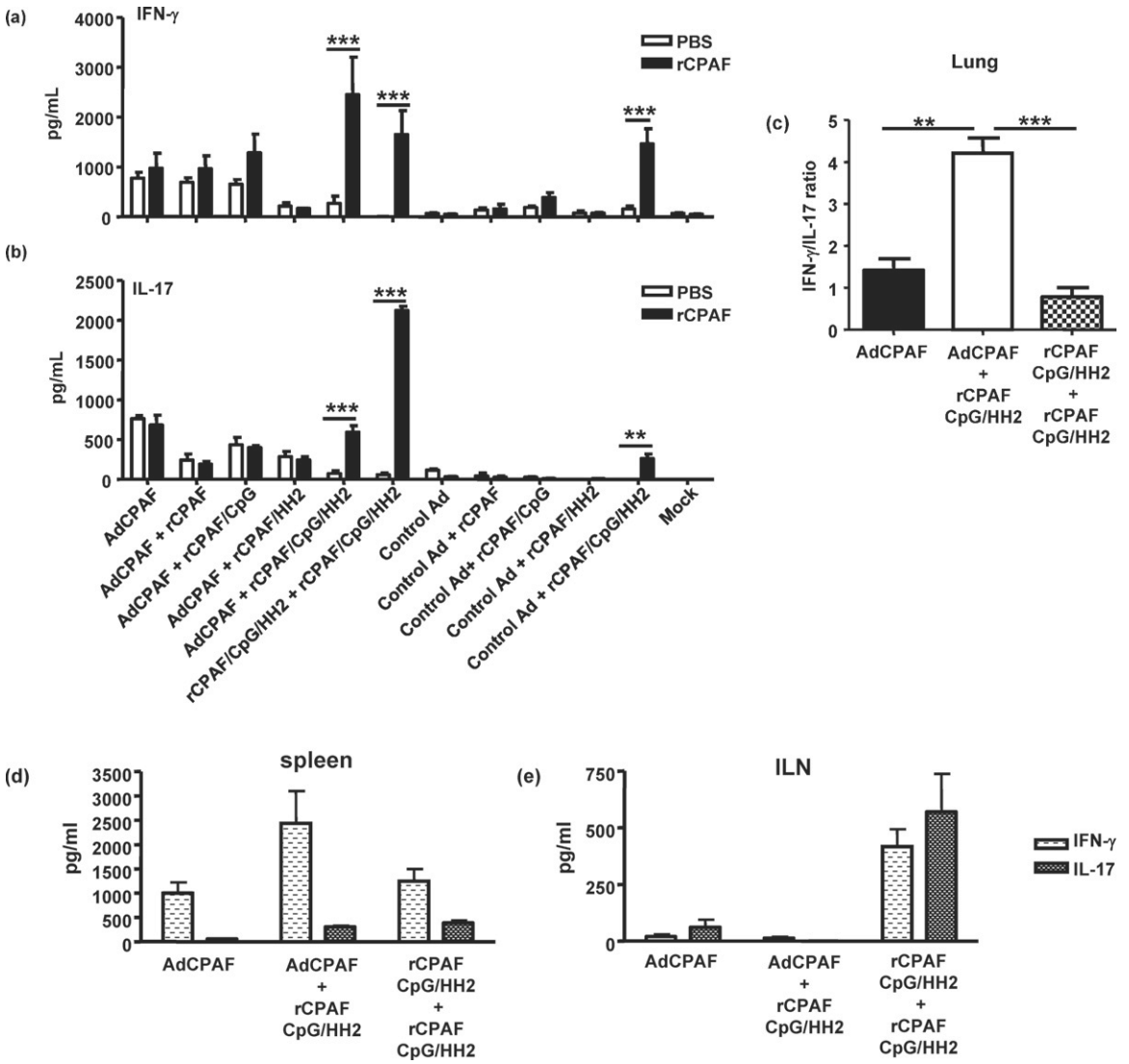

Fig. 4. Cellular immune responses triggered by different vaccination regimens. C3H/HeN mice were immunized i.n. with various vaccination regimens as indicated and sacrificed four weeks after the booster immunization. Leukocytes were isolated from lung (a–c), spleen (d) or ILN (e) and stimulated with or without recombinant CPAF for 72 h. The content of IFN-γ and IL-17A in the culture supernatant was measured by ELISA. Data are presented as mean ± SEM (n = 3–4 per group). Student's t-test was used to compare CPAF-stimulated culture condition relative to unstimulated condition. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

prime-boost immunization regimen displayed strong Th1 and Th17 responses in the ILN. Similar immune profile was also observed in BALB/c mice (data not shown).

3.5. Homologous and heterologous prime-boost vaccination regimens confer significant immune protection against Chlamydia genital challenge

Having demonstrated that different i.n. vaccination regimens stimulated distinct profile of humoral and cellular immune responses, we sought to understand how different profiles of immune response correlate with the protective efficacy against genital *Chlamydia* infection. We selected vaccination regimens of a single dose of AdCPAF, the heterologous prime-boost regimen (AdCPAF + rCPAF/CpG/HH2) and the homologous prime-boost regimen (rCPAF/CpG/HH2 + rCPAF/CpG/HH2) for further evaluation in both C3H/HeN and BALB/c mice. As shown in Fig. 5, C3H/HeN mice immunized with a single dose of AdCPAF had a significantly reduced bacterial load in the vagina at day 6 post challenge compared to control Addl-immunized mice, yet, no protection was observed at any other time points. In comparison, mice receiving the heterologous prime-boost regimen showed a significant immune protection on days 12, 18 and 24-post challenge compared to Ctrl Ad-primed group and/or mock-immunized mice. Significant immune protection was also observed in mice immunized with the homologous prime-boost immunization regimen on days 12 and 18-post challenge compared to mock-immunized mice. Similar data was also observed in BALB/c mice (data not shown). Histological analyses showed that the rate of hydrosalpinx development in C3H/HeN mice including mock-immunized mice was low (data not shown), whereas 90% of mock-immunized BALB/c developed hydrosalpinx (Fig. 5b). Both the heterologous prime-boost and the homologous

ND = not detectable, $^{*}p \leq 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (g) The ratio of IgG2a to IgG1 for each mouse. The line indicates the median ratio of IgG_{2a} to IgG_1 within the group. (h–i) CPAF-specific total antibody (h) and IgA (i) levels in the genital tract. Results are presented as the mean ± SEM (n = 4–7 mice per group). A one-way ANOVA test followed by a Post-Hoc Tukey's multiple comparison test was used for statistical analysis. $^{*}p \leq 0.05$.

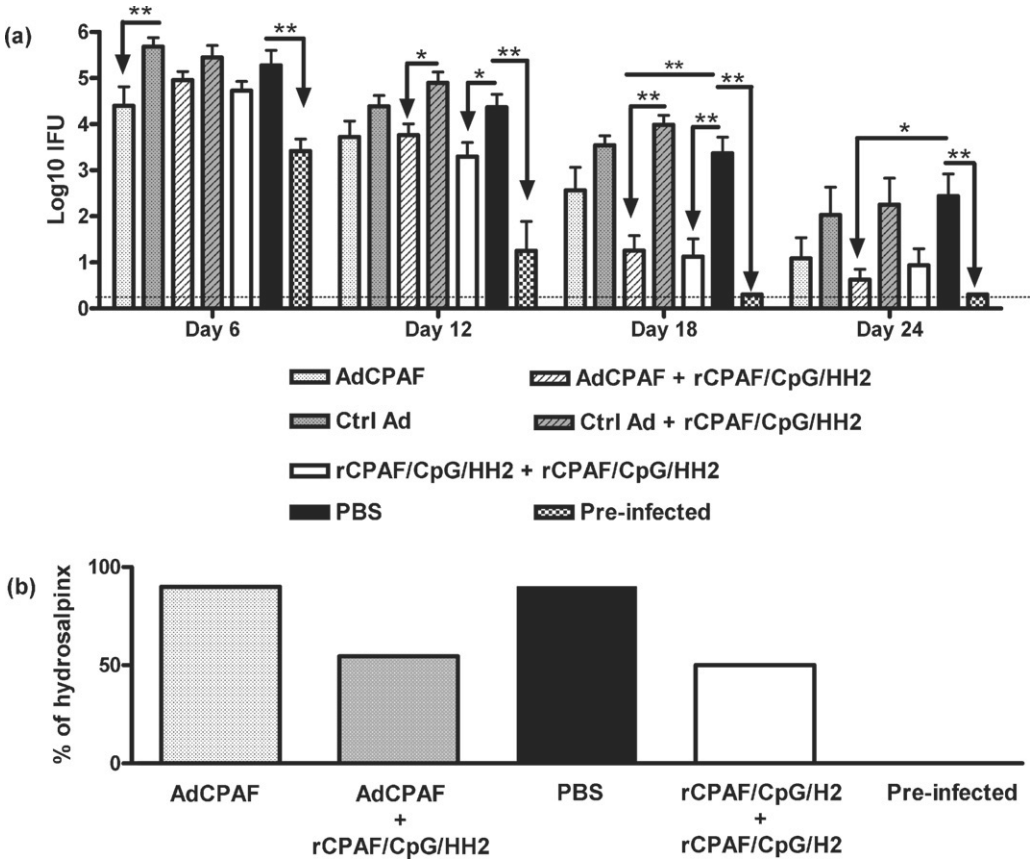

Fig. 5. Immune protection against genital *Chlamydia* challenge in C3H/HeN mice immunized with different vaccination regimens. (a) C3H/HEN mice were immunized i.n. with various vaccination regimens as indicated. Four weeks after booster immunizations mice were challenged intravaginally with 5×10^4 IFU of *C. muridarum*. The bacterial recovery in the genital swabs at days 6, 12, 18 and 24 post-infection. Line indicates mean IFU within the group ($n = 10$–12 per group for all testing groups, $n = 4$ for pre-infected group). One-way ANOVA test was used for statistical analysis. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$; $^{***}p \leq 0.001$. (b) The percent of hydroslpinx in BALB/c mice ($n = 10$–12 mice) as assessed on day 50-post *C. muridarum* challenge.

prime-boost regimens were able to reduce the incidence of hydrosalpinx to ~50% (Fig. 5b).

Together, our results demonstrate that intranasal immunization regimen with a single dose of AdCPAF only triggered limited protection against genital *C. muridarum* infection whereas the heterologous and the homologous prime-boost immunization regimens using CpG/HH2 complex as adjuvant were considerably more protective. These data, in conjunction with the profile of humoral and cellular immune responses observed in mice immunized with different vaccination regimens, further support a critical role of T-cell immunity in regulating *Chlamydia* vaccine efficacy.

4. Discussion

In this study, we examined the immune responses and protective efficacies of different intranasal vaccination regimens in murine *C. muridarum* infection models. A single dose of AdCPAF that stimulated robust humoral immunity but weak cellular immune responses was found to provide limited immune protection against *C. muridarum* challenge in mice. In comparison, the heterologous prime-boost regimen using AdCPAF for priming and a recombinant CPAF subunit vaccine formulated with CpG and HH2 complex for boosting stimulated strong humoral and cellular immune responses with characteristics of a Th1-biased profile. In contrast, a homologous regimen using rCPAF/CpG/HH2 subunit vaccine for both priming and boosting induced a weak antibody response, but potent cellular immunity with a mixed Th1/Th17 profile. Despite such differences in Th1/Th17 profile, both the heterologous and homologous prime-boost regimens conferred significant immune protection against genital *C. muridarum* challenge with comparable efficacies in C3H/HeN and BALB/c mice.

Both IFN-γ/Th1 and IL-17/Th17 immune responses are involved in host resistance against *Chlamydia* infection [1,10,11]. In our study, a single dose of AdCPAF intranasal delivery only stimulated a weak CPAF-specific Th1 and Th17 immune profile. However, a booster rCPAF subunit vaccine formulated with CpG/HH2 complex, but not CpG alone or HH2 alone, markedly increased Th1 immune response. While this observation is in line with previous studies demonstrating that CpG and HH2 have synergistic adjuvant effects [27,28], our data revealed an unidentified potent adjuvant effect of CpG/HH2 complex on cellular immune responses. Of interest, two consecutive i.n. deliveries with rCPAF/CpG/HH2 stimulated a robust Th17 response mixing with a Th1 response. While these results are in agreement with a recent study demonstrating that i.n. immunization with a soluble protein preferentially induces a Th17 response [25], it is likely that the immune responses induced by AdCPAF priming have some negative impact on the development of Th17 response upon boosting with rCPAF/CpG/HH2. The precise molecular pathway in mediating this response is unclear at this moment.

It was unexpected that the heterologous and the homologous prime-boost regimens conferred comparable levels of protective efficacy given the understanding that Th1 and Th17 responses cross-regulate each other. IFN-γ is known to inhibit Th17 differentiation [41,42], and IL-17 is also reported to directly suppress Th1 cells [43,44] or to promote Th1 immunity [11,45] depending on the experimental conditions. Thus, given that both IFN-γ/Th1 and IL-17/Th17 responses are critical determinants of anti-*Chlamydia*

immunity, the interplay between Th1 and Th17 response is inevitably involved in regulating host resistance to *Chlamydia* infection. In this regard, *Chlamydia*-susceptible mouse strains (C3H/HeN and BALB/c mice) are found to develop more heightened IL-17/Th17 responses than *Chlamydia*-resistant counterparts (C57BL/6 mice) [8,12], suggesting that over-production of IL-17/Th17 likely compromises host resistance to *Chlamydia* infection. Therefore, the relative strength of Th1 to Th17 immunity is likely to play a role in regulating *Chlamydia* vaccine efficacy. In our study, however, the homologous prime-boost regimen induced a comparable level of immune protection in C3H/HeN and BALB/c mice compared to its heterologous counterpart. It is likely that, in addition to Th1/Th17 profile, other factors are also involved in regulating *Chlamydia* vaccine efficacy. A drastic difference in the distribution of cellular immunity in ILN was observed in mice receiving the heterologous and the homologous prime-boost regimens (Fig. 4e). While i.n. immunization is widely recognized as a novel immunization method for inducing antigen-specific immune responses at far distant genital mucosal site, our data suggest that this notion is more applicable for antibody responses. In the case of cellular responses, the vaccine platform appears to play a critical role in determining the distribution and migration of memory T cells. It is likely that memory T cells induced by i.n. immunization with rAd-based vaccines are preferentially accumulated in the lung – the site of immunization, but not in the ILNs – the site draining the genital tract. This observation is indeed in agreement with previous studies [17,18,46], although it contradicts with others [23,47]. Of interest, despite the low level of memory responses in the ILN of mice receiving the heterologous prime-boost regimen, these mice were significantly protected from the challenge. We believe that the quality of memory T cells induced by the heterologous prime-boost regimen is superior to other vaccination regimens although the long-distance travel of memory T cells upon *C. muridarum* challenge in these mice offsets the host resistance at the genital tract. Therefore, although a clear understand about the role of Th1/Th17 profile in regulating vaccine efficacy needs to be further analyzed in other studies, this study provides clear evidence that i.n. immunization with rAd-based vaccine is more suitable for respiratory infections than for STI. An improved heterologous prime-boost regimen using AdCPAF may need to involve intra-vaginal immunization to trigger potent protective humoral and cellular immunity at genital tract as suggested by another study [48].

Antibodies also mediate protective immunity against secondary *Chlamydia* infection. Adoptive transfer of antibody-containing sera or purified monoclonal antibodies that recognize *Chlamydia* membrane protein MOMP or LPS displayed significant protective efficacy against secondary *Chlamydia* challenge [3,49]. Previous studies demonstrated that CpG/HH2 complex has potent adjuvant activities in stimulating B cell proliferation and antibody production relative to CpG alone or HH2 alone [27,28]. In our study, however, a single dose of AdCPAF stimulated high titers of antibody production, which were significantly better than that induced by the rCPAF subunit vaccine formulated with CpG/HH2 complex. These results indicate that viral-based vaccines are more effective than subunit vaccines at stimulating antibody production. However, the homologous vaccination regimen was more efficacious than a single dose of AdCPAF despite superior CPAF-specific humoral immunity induced by a single dose of AdCPAF. Thus, our data suggest that CPAF-specific antibody has a limited role in contributing to the protective immunity induced by our vaccination regimens. The possible explanation is that CPAF is a cytosolic protein, which cannot be easily accessed by anti-CPAF antibody. Consistent with this idea, adoptive transfer of purified monoclonal antibody recognizing cytosolic protein heat-shock protein (HSP)-60 also failed to confer immune protection despite high titers of anti-HSP-60 in the genital tract upon adoptive transfer [49]. Thus, although anti-CPAF antibodies do not seem to contribute to anti-*Chlamydia* immunity in our study, it is reasonable to propose that an optimized heterologous prime-boost vaccination regimen can be developed by utilizing a bivalent-antigen consisting of CPAF and a surface antigen such as MOMP for stimulating protective humoral and cellular immunities.

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