

A Novel LNP-based *Chlamydia* subunit vaccine formulation that induces Th1 responses without upregulating IL-17 provides equivalent protection in mice as formulations that induced IL-17 and Th1 cytokines

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Research article

Keywords: LNP, Chlamydia, IL-17, mouse model, Th1 cytokines

Posted Date: November 4th, 2019

DOI: <https://doi.org/10.21203/rs.2.16752/v1>

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Abstract

Sexually transmitted Chlamydia infections can lead to the development of debilitating diseases such as chronic pelvic pain, ectopic pregnancy, pelvic inflammatory disease, and infertility. It has been proposed that immunity against Chlamydia infection and disease may be determined by a balance of IL-17 signaling. We sought to evaluate novel formulations for a candidate Chlamydia vaccine, consisting of Chlamydia major outer membrane protein (MOMP) alone or in combination with polymorphic membrane protein D (PmpD) and polymorphic membrane protein G (PmpG) as target immunogens. Native MOMP (nMOMP) isolated from *C. muridarum* elementary bodies (EBs) and recombinant PmpD and PmpG proteins were adjuvanted with Monophosphoryl lipid A (MPLA), in one of two formulations containing either lipid nanoparticles (LNPs) or the cationic lipid dimethyldioctadecylammonium bromide (DDA). Antibody titers to *C. muridarum*, nMOMP, and EBs were evaluated by ELISA, and T-cell responses by intracellular cytokine staining (ICS). Protection from challenge was determined by qPCR and gross pathology. All mice immunized with the new vaccine formulations showed significantly higher antibody titers to nMOMP ($P < 0.001$) and *C. muridarum* EBs ($P < 0.001$), when compared to the negative control group (adjuvant alone). Antibody titers in vaccine groups with Monophosphoryl lipid A (MPLA)+LNP were higher as compared to the MPLA+DDA group ($P < 0.001$) except for groups 6 (Cm nMOMP+PmpG+PmpD p73+PmpD p82+MPLA+DDA) vs 7 (Cm nMOMP+PmpG+PmpD p73+PmpD p82+MPLA+LNP) for both *C. muridarum* EBs and PmpG; the groups were not statistically significant. ICS analysis showed more robust CD4+ T-cell responses (IFN- γ /IL-2/TNF- α) in the dimethyldioctadecylammonium bromide (DDA) and LNP groups compared to the adjuvant alone group. The combination of DDA and MPLA gave robust Th17 responses in comparison to MPLA and LNP group. Immunized groups also showed protection from challenge with *C. muridarum*, as evidenced by a reduction in bacterial shedding from the vagina for all groups ($P < 0.003$) compared to shedding from the adjuvant control, Group 1. Both vaccine formulations generated robust immunological responses and both vaccine formulations were protective by reducing bacterial shedding after challenge. This data indicates equal protection can be achieved without the induction of Th17 responses.

Background

Chlamydia infections cause substantial morbidity and socio-economic burden world-wide, and although antibiotic treatment is effective, the high rate of asymptomatic infections (70% in women) leads to chronic infections that may ultimately cause upper genital tract pathology. An efficacious vaccine could prevent the disease sequelae that are common following *Chlamydia* infections including infertility and ectopic pregnancy (1–3). Each year approximately 1.4 million new cases of sexually transmitted *C. trachomatis* infections are diagnosed in the United States and approximately 113 million new cases are reported worldwide (2, 4). Although *C. trachomatis* infection is responsive to antibiotic treatment, more than 70% of infections in women are asymptomatic (5), making both diagnosis and control of infection difficult. Therefore, there is a substantial need for an efficacious vaccine to protect against *Chlamydia* infection.

Chlamydia vaccine development has been decades long endeavor. In recent years encouraging progress has been made towards a native MOMP based vaccine with levels of protection approaching those of live EB vaccination in mice (6–8). An antigen based subunit vaccine, whether using native purified proteins or recombinantly expressed proteins appears as a practical path forward. Although MOMP is a promising vaccine target, additional antigens may need to be included to create an efficacious vaccine.

Recent vaccine studies using MOMP and Pmp combinations have shown promise as vaccine candidates with early clearance of *Chlamydia* infections compared with unvaccinated control groups (9–12). The Pmp family of proteins are good T-cell antigens and potential targets for a T-cell focused vaccine (9, 11, 13). Native MOMP has been shown to elicit a strong humoral immune response (14). A vaccine that includes antigens that provide both a T-cell and antibody response against *Chlamydia* may provide optimal protection against both initial and repeat infection than a vaccine profile that only elicits a Th1 or Th2 responses (11). IL-17 mediated immunity has been shown to play a role in the control of intracellular pathogens, including *Chlamydia* (10, 15) However, there is also evidence for a pathogenic role through enhanced recruitment of polymorphonuclear neutrophils and inflammatory monocytes in the UGT. The Th17-mediated inflammation has been associated with tissue destruction, intra-abdominal adhesions and increases in splenic weights in the mouse model (15–17). We tested the role of Th1 and/or Th17 T-cells in affording protection from *Chlamydia* infection and their ability to cause pathology in the upper genital tract utilizing two vaccine formulations.

We have evaluated numerous conserved *Chlamydia* antigen targets (data not shown) with minimal protection or reduction in bacterial burden observed following immunization and intravaginal challenge from most targets. Interestingly, we have observed protection with vaccine formulations including nMOMP similar to what has been published in the literature (6–8, 18). Since MOMP is a good humoral antigen, we also looked for antigens that would elicit cell-mediated immune responses. Immunoproteomic vaccine studies found that four polymorphic membrane proteins (PmpE, PmpF, PmpG and PmpH) are immunodominant, recognized by various MHC class II haplotypes and are protective in mouse models (9–11). PmpG/F have been shown to be the most immunodominant of the Pmps (11). Therefore, based on protection with nMOMP observed in our laboratory; and publications citing protective immunity for both MOMP and polymorphic membrane proteins, with PmpG/F being most immunodominant of the pmps, we focused on nMOMP, PmpG, PmpD passenger domain (PmpD-p73) and PmpD translocator domain (PmpD-p82) as candidate vaccine targets in the current study. Additionally, various adjuvants were included to drive the cell mediated immune response toward Th1, and/or Th17 and the role of T-helper population in conferring protection was evaluated. To determine the role of Th17 mediated immunity, vaccine formulations in some test groups were adjuvanted with the cationic lipid dimethyldioctadecylammonium bromide (DDA) which has been shown to induce potent Th1 and Th17 responses (19) whereas the lipid nanoparticles (LNP) formulations were used to bias toward Th1 responses. Immunogenicity was evaluated for both T-cell responses and antibody, and efficacy was determined by measuring the magnitude and duration of shedding of *Chlamydia* after intravaginal challenge. The combination of DDA and MPLA gave robust Th17 responses in comparison to LNP and MPLA. Both sets of formulated antigens (MPLA+LNP or MPLA+DDA) resulted in robust antibody titers to

nMOMP ($P < 0.001$) and *C. muridarum* EBs ($P < 0.001$), when compared to the adjuvant-alone control group. Immunized groups also showed protection from challenge with *C. muridarum*, as evidenced by a reduction in bacterial shedding from the vagina for all groups ($P < 0.003$) compared to shedding from the adjuvant control, Group 1. Taken together, the data suggest that the vaccines formulated with either MPLA+LNP or MPLA+DDA elicited strong immunological responses and both were protective by reducing bacterial shedding after challenge. Furthermore, equal protection is achieved with or without a Th17 response.

Methods

Ethics statement

All animal experiments were approved by the Merck & Co., Inc., Kenilworth, NJ, USA, Institutional Animal Care and Use Committee (IACUC). All procedures were performed in accordance with our institution's IACUC guidelines in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. Mice were housed in large mouse containers ($n = 10$ mice/box and $n = 5$ mice/box for ICS) with micro isolator lids, and the rooms were maintained with controlled humidity and temperature, and 12-hour light-dark cycles. All containers had nestlets and animals were provided standard chow (Purina 5001 rodent diet) and water ad libitum. The physical condition of the animals was monitored daily (7 days a week), and any health changes were noted. At the end of the study, animals were euthanized using CO₂ inhalation (10% - 30% CO₂ in the air mixture inhaled per minute). No animals were found to be moribund or to have any adverse events throughout the study.

Cell culture and propagation of Chlamydia muridarum

C. muridarum was obtained from Bernard Arulanandam (UTSA San Antonio, Texas). HeLa 229 cells were used for propagation of *C. muridarum*. HeLa 229 cells were grown in Eagle's Minimal Essential Medium (EMEM; ATCC, Manassas, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT), 50 µg/ml vancomycin (Sigma, St. Louis, MO), and 10 µg/ml gentamicin (Gibco; Carlsbad, CA). Host cells were seeded into tissue culture flasks at a cell density of 5×10^5 cells/ml and incubated overnight at 37°C in 5% CO₂ to achieve a confluent monolayer. Cell monolayers were infected with a *Chlamydia* stock diluted in sucrose-phosphate-glutamate (SPG) buffer. The *C. muridarum* strain was cultured for 48 hours. The *Chlamydiae* were harvested from the infected cells and purified by centrifugation through 30% Renograffin (Bracco Diagnostics, Princeton, NJ) and stored frozen at -80°C.

Chlamydia recombinant proteins

The recombinant *Chlamydia* proteins were cloned, expressed and purified as follows: PmpG (amino acids 25–500), PmpD passenger domain (p73) (amino acids 53–757) and PmpD translocator domain (p82) (amino acids 758–1520) as described by Swanson (20); *C. muridarum* gene sequences were synthesized by Genart (Thermo Fisher, Germany) and cloned into pET30a and pET28a vectors (Novagen, Cambridge, MA), respectively, using standard molecular biology techniques. Plasmids containing the

genes were transformed into the *Escherichia coli* strain BL21(DE3) (New England Biolabs, Ipswich, MA) with protein expression induced using the lac promoter for T7 RNA expression using isopropyl- β -D-thiogalactopyranoside (IPTG).

Purification of His-tagged PmpG, PmpD-p73 and PmpD-p82

Cell pellets of *E. coli* expressing PmpG, PmpD-p73 or PmpD-p82 were resuspended in lysis buffer (50 mM TRIS, pH 8.0, 300 mM NaCl, and 1X protease inhibitors (Roche, Basel, Switzerland, EDTA free), and lysed with three passes through a microfluidizer (Microfluidics M-110P) at 15,000psi. The lysate was clarified by centrifugation (~19,000xg) and the pellet (containing inclusion bodies) was solubilized in a urea buffer (8M urea, 10mM MES pH 6.5, 150mM NaCl, and 20mM Imidazole) and clarified by ultracentrifugation (~160,000xg). The solubilized fraction was purified while in urea by immobilized metal affinity chromatography (IMAC) with Ni NTA agarose and step eluted with 200mM imidazole. The IMAC product was refolded using extensive dialysis against 10mM MES pH 7.3, 150mM NaCl containing 0.1% Zwittergent, 3-14 (PmpD-p73 and PmpD-p82) or phosphate buffered saline (PBS) containing 0.1% Zwittergent, 3-14 (PmpG) using a Pierce Slide-A-Lyzer (Pierce, Rockford, Illinois). cassette with a 10 kDa MWCO while step reducing the urea concentration to zero. The PmpD-p73 and PmpD-p82 in the MES buffered saline containing 0.1% Zwittergent, 3-14 or the PmpG in the PBS containing 0.1% Zwittergent, 3-14 were used in both *in vitro* and *in vivo* studies. The final products were sterile filtered and the concentration was determined by amino acid analysis. Purity of the final product was evaluated by SDS-PAGE and by in-solution enzymatic digestion with 1D/LC-MS-MS analysis.

Amino Acid Analysis

Vapor phase hydrolysis was performed with constant boiling 6 N HCl at 150 °C for 1 hour using an ELDEX (Napa, CA) H/D workstation. Protein hydrolysates and amino acid standards were derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) using the Waters AccQ Tag Ultra derivatization kit according to the manufacturer's protocol. AQC reacts with primary and secondary amines to form stable fluorescent derivatives that can be separated by RP-HPLC. The fluorescent AQC derivatives were separated on an AccQ Tag Ultra C18, 1.7 μ m, 2.1 x 100 mm column using a modified gradient program.

Native MOMP purification from infected cells (HeLa)

Native MOMP (nMOMP) was purified by extraction using sonication of intact *Chlamydia*-infected HeLa cells based on a method by de La Maza (18) with modifications to infected cell starting material, less frequent sonication, and altered chromatography. The initial steps used phosphate buffer with CHAPS and 100mM DTT and subsequently a phosphate buffer containing Zwittergent, 3-14 with 100mM DTT. The MOMP was further purified post-extraction with hydroxyapatite chromatography. The hydroxyapatite product was concentrated using tangential flow filtration (TFF), and then purified with anion exchange chromatography. The nMOMP was dialyzed into 10mM MES pH 7.3, 150mM NaCl, and 0.1% Zwittergent, 3-14 for use in animal dosing and *in vitro* studies.

Mice

Female C57BL/6 mice were purchased from Taconic Farms (Hudson, NY). At the time of study start, mice were 6–7 weeks of age, with food and water provided *ad libitum*. Animals were randomly assigned to cages upon receipt from the vendor. All animal experiments in these studies were approved by the Institutional Animal Care and Use Committee (IACUC), Merck & Co., Inc., West Point, PA, USA. All procedures were performed in accordance with our institution's IACUC guidelines in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. Animals were treated and assessed in numerical order groups 1–8.

Murine model and specimen collection

Study design is shown in *Figure 1*. Fifteen animals per group (10 for challenge and 5 for ICS) were immunized by subcutaneous (s.c.) route with the following combinations of adjuvants and antigens as seen in *Table 1*: Group 1) MPLA+ DDA+ LNP (adjuvant control), Group 2) Cm nMOMP+MPLA+DDA, Group 3) Cm nMOMP+MPLA+LNP, Group 4) Cm nMOMP+PmpG+MPLA+DDA, Group 5) Cm nMOMP+PmpG+MPLA+LNP, Group 6) Cm nMOMP+PmpG+PmpD p73+PmpD p82+MPLA+DDA, Group 7) Cm nMOMP+PmpG+PmpD p73+PmpD p82+MPLA+LNP, Group 8) Live Cm EB (positive control) were immunized with 1×10^6 EB in SPG per mouse by intraperitoneal (i.p.) route.

Table 1. Vaccine Groups

Group	Vaccine	Antigen Dose (ug)	MPLA Dose	DDA Dose	LNP Dose
1	MPLA+DDA+LNP	-	5 ug	250 ug	100 ug
2	Cm nMOMP+MPLA+DDA	10	5 ug	250 ug	-
3	Cm nMOMP+MPLA+LNP	10	5 ug	-	100 ug
4	Cm nMOMP+PmpG+MPLA+DDA	10/10	5 ug	250 ug	-
5	Cm nMOMP+PmpG+MPLA+LNP	10/10	5 ug	-	100 ug
6	Cm nMOMP+PmpG+PmpD p73+PmpD p82+MPLA+DDA	10/5/5/5	5 ug	250 ug	-
7	Cm nMOMP+PmpG+PmpD p73+PmpD p82+MPLA+LNP	10/5/5/5	5 ug	-	100 ug
8	Cm EBs	1×10^6 IFU	-	-	-

Tail-vein bleeds were performed on days –1 and day 42. Immunizations were performed on days 0, 20, 30. Spleens were harvested on day 40 from 5 mice per group. Mice were rested for 1 month prior to intravaginal challenge. Progesterone [medroxyprogesterone acetate (Depo-Provera; Pfizer; New York, NY)] was administered subcutaneously (2.5 mg/dose) on days 45 and 52 of the study prior to challenge. Mice were anesthetized in their home cage prior to challenge only with a mixture of 85 mg/kg ketamine (Ketaset; Fort Dodge Animal Health, Fort Dodge, IA) and 5 mg/kg xylazine (AnaSed® Injection; Henry Schein Animal Health, Dublin, OH) by i.p. route. Mice were challenged intravaginally by direct instillation of 5,000 IFU *C. muridarum* in 10 µl SPG on Day 55. The course of infection was monitored by enumerating *Chlamydiae* recovered from cervicovaginal swabs collected on days 62, 69, 76. The vaginal vault and

ectocervix were swabbed using a microfiber swab (Fisher; Pittsburgh, PA). Swabs were placed into a 1.5 ml tube containing two sterile glass beads (5 mm diameter) and 300 µl of *Chlamydia* Isolation Medium (Trinity Biotech; Berkeley Heights, NJ) on ice. Bacteria were eluted from the swabs and separated from cells by vortexing for 60 seconds. Eluted bacteria were split into two aliquots and stored at -70°C until DNA extraction.

At spleen harvest or at the end of the study, animals were euthanized using CO₂ inhalation (10% - 30% CO₂ in the air mixture inhaled per minute). No animals were found to be moribund throughout the study. Gross pathology was assessed on day 118.

Primer, probe and real-time quantitative PCR

The qPCR assays were performed as described in Wooters et al. (21), with the following modifications. Briefly, DNA from genital swab samples (~100 µl) was extracted using the MagNA Pure 96 DNA and Viral NA small volume kit (Roche, Basel, Switzerland) on the MagNA pure machine (Roche, Basel, Switzerland) according to the manufacturer's instructions. The oligonucleotide primer set was designed for detection of all species of *Chlamydiae*. The sense primers, 16S DIR 5'-ATG CAT ATG ACC GCG GCA A-3', and 16S DIR 5'-ATG TAT ATG ACC GCG GCA GA-3' and anti-sense primer, 16S Rev 5'-TAA GGG CCA TGC TGA CTT GA-3', were designed to amplify a 508-bp fragment of the *Chlamydia* 16S ribosomal subunit gene, conserved across *Chlamydia* strains and serovars. Primers were obtained from Sigma-Aldrich (St. Louis, MO), and the probe, 16S Fam-5'-TTA ACC AGG AGG AAG GCG AG-3' Tamra, was also synthesized by Sigma-Aldrich (St. Louis, MO). The 50-µL reaction mixtures consisted of 1X QuantiTect Multiplex PCR master mix without ROX (Qiagen, Hilden, Germany), 100 nmol/L 16S probe, 200 nmol/L primer 16S DIR, 400 nmol/L primer 16S Rev, 30 nmol/L ROX reference dye, and 5 µL of sample DNA. Nontemplate controls consisting of the reaction master mix, primers, and probe, but no DNA, were included in each assay run. Reaction conditions were set as follows: 1 cycle at 95°C for 15 min, followed by 40 cycles at 94°C for 1 min and at 60°C for 1 min. Thermal cycling, fluorescent data collection, and data analysis were performed using the Stratagene Mx3005P system (Stratagene, San Diego, CA) according to the manufacturer's instructions. Data were plotted as Area Under the Curve (AUC) for individual mice and further analyzed by Wilcoxon Rank Sum Test (compared to MPLA / DDA / LNP).

Detection of serum antibody and isotype levels by ELISA

Serum was analyzed by an enzyme-linked immunosorbent assay (ELISA). Nunc™ C96 Maxisorp Immunoplates (Thermo Scientific, Waltham, MA) were coated with 50 µL of 1 µg/ml *C. muridarum* EBs in PBS at 4°C overnight. The plates were washed three times with PBS containing 0.05% Tween-20 (Fisher Scientific, Hampton, NH)(PBS-T). The wells were blocked with 5% Fetal Bovine Serum Albumin (BSA) (Thermo Scientific, Waltham, MA) in PBS-T at 200 µL/well for 1 hour at room temperature and washed three times with PBS-T. Serum was diluted in 5% FBS in PBS at a 1:500 dilution. Serially diluted sera were added to the plate, incubated for 2 hours at room temperature and were washed three times with PBS-T. HRP-conjugated secondary antibodies (Goat anti-mouse IgG, Fcγ fragment specific 1:6,000; Goat anti-mouse IgG, Fcγ Subclass 1 specific 1:6,000; or Goat anti-mouse IgG, Fcγ Subclass 2c specific 1:2,000;

Jackson ImmunoResearch Laboratories, Grove, PA) were diluted in 5% FBS in PBS. The diluted secondary antibodies were added at 100 μ L/well, incubated for 1 hour at room temperature and were washed three times with PBS-T followed by three times with PBS. Room temperature BD Opt EIA™ TMB Substrate Reagent Set (BD Biosciences, Franklin Lakes, NJ) was mixed and filtered through a 0.22 μ m cellulose acetate (CA) filter unit (Corning, Corning NY), and 100 μ L was added to each well and incubated for 10 min at room temperature. The reaction was stopped with 100 μ L/well of 2M H₂SO₄ (Fisher Scientific, Hampton, NH). The optical density (OD) was read at 450 nm on a SpectraMax® M5 (Molecular Devices, San Jose, CA). The cutoff OD for each post-immunization serum was calculated as two times of the OD₄₅₀ of the corresponding pre-immunization serum. ELISA titers were determined by linearly interpolating between the sequential log dilutions that bracket the cutoff OD, where the dependent variable is the OD response and the independent variable is the log dilution. The resulting dilution is then back transformed to obtain the reported titer. The reported titer is the estimated dilution of serum that results in a response equivalent to the endpoint OD. Transformed was data analyzed by One-way ANOVA with Dunnett post test

T-cell intracellular cytokine staining

Ten days post final immunization, spleens from vaccinated animals were collected and processed to isolate splenocytes. One million splenocytes/well were incubated with 2 μ g/ml of specific peptide pools for MOMP, PmpG or PmpD (15mer overlapping by 11, custom order JPT Peptide Solns, Germany) in the presence of Brefeldin A and anti-mouse CD28 and CD48b antibodies (BD Biosciences, Franklin Lakes, NJ). As a negative control, a sample for each was set up with a matched volume of DMSO and costimulatory antibodies, 5 hours post incubation at 37°C, splenocytes were incubated with mouse FC block (BD) surface stained with viability dye (Thermo Fisher, Waltham, MA) anti-mouse-CD3 (BD), CD4 (BD), CD8 (BD) followed by permeabilization and intracellular staining for anti-mouse IFN γ (BD), TNF α (BD), IL-2 (BD), and IL-17A (Biolegend), San Diego, CA). Fixed samples were then run on FACS LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ) and data were analyzed using FloJo software (Treestar Inc., Ashland, OR). The data analysis program Simplified Presentation of Incredibly Complex Evaluations (version 4.1.6; provided by M. Roederer, Vaccine Research Center, NIAID, NIH) was used to generate the graphical results in Figure 2. All peptide stimulated responses were reported after subtraction of the unstimulated controls. Responses were compared to the adjuvant only group (MPLA+DDA+LNP) in a two tailed paired t test.

Results

Recombinant and native protein purification and analytical evaluation

PmpD (separated into the passenger domain p73 and tranlocator domains p82) and PmpG were cloned and expressed using pET vector systems. The recombinant proteins were expressed in the inclusion body fraction of IPTG-induced BL21DE3 cells. The inclusion body fractions were denatured and recombinant proteins were purified and refolded (made soluble) using dialysis refolding techniques that provided high

purity proteins for *in vitro* and *in vivo* use. All antigens tested had purities of >99% by 1D/LC-MS-MS analysis and were maintained in buffers (PBS with 0.1% Zwittergent,3–14 or MES/NaCl with 0.1% Zwittergent,3–14) that were suitable for both *in vitro* and *in vivo* studies. Native MOMP (nMOMP) was purified from large scale preps of *Chlamydia muridarum* infected HeLa cells. The nMOMP materials used in these studies were analyzed by amino acid analysis to determine concentration and then by 1D/LC-MS-MS analysis (purity >95%), size exclusion chromatography with multi-angle light scattering detection to determine structure (predominantly trimer with some monomer), circular dichroism to determine form (~40% Beta-sheet structure), and SDS-PAGE analysis to confirm purity and structural characteristics (data not shown).

Immunization with Chlamydia proteins adjuvanted with LNP or DDA induced robust and distinct polyfunctional CD4+ T-cell response profiles, and IL–17 did not enhance protection from challenge

Due to the well-established connection between CD4+ T-cells and control of *Chlamydia*, we included potent T-cell adjuvants in our vaccine formulations (22–25). To explore the role of IL–17 in protection from *Chlamydia* challenge, we included formulations containing the cationic lipid, DDA, which was shown to be a potent promoter of IL–17 in previous vaccine studies (10)). Multiparameter flow cytometry was performed to evaluate antigen specific IFN- γ , TNF-, IL–2 and IL–17 producing T-cells in splenocytes ten days after the final immunization (d40). Five mice per group were analyzed for ICS.

Robust MOMP specific CD4+ T-cells were observed in groups vaccinated with adjuvanted nMOMP (Figure 2). Furthermore, MOMP responses of similar magnitude were detected from mice that were vaccinated with additional proteins (PmpG, PmpD) (Figure 2), demonstrating a lack of immune interference.

Strong CD4+ T-cell responses specific for PmpD or PmpG were also detected in mice vaccinated with the respective adjuvanted proteins (Figure 2). Surprisingly, despite the increased protection afforded by the intraperitoneal *C. muridarum* elementary body immunization (Figure 4), mice in these groups demonstrated low levels of CD4+ T-cell responses in the spleen when stimulated with MOMP, PmpD and PmpG peptides. Compared to the adjuvant only control group, significant T-cell responses (IFN- γ and TNF-) in EB exposed mice were only detected towards MOMP and PmpG (Figure 2). Additionally, these responses were much lower in magnitude as compared with the subcutaneous protein vaccinated groups.

As expected, potent IL–17 responses were detected from mice in groups that were vaccinated with DDA containing formulations (Figure 2D), and such responses were not induced by the LNP formulations or *C. muridarum* EB exposure. Additionally, IL–2 cytokine production trended higher in the groups which received DDA (Figure 2C).

As polyfunctional T-cell responses have been shown to correlate with protection in many infectious disease models, we evaluated the percent of total responses in each group which produced all four cytokines (IFN- γ /IL–2/TNF-/IL–17), any combination of the three, two or any one cytokine (Figure 2). Primarily due to significantly increased IL–17 and IL–2 production in the DDA adjuvanted vaccine groups

(Figure 2C and 2D), a higher percentage of polyfunctional T-cells were detected in the DDA adjuvanted vaccine groups as compared to the LNP or EB exposed groups (Figure 2E).

Vaccine immunizations result in high ELISA titers to nMOMP, C. muridarum EBs, PmpG and PmpD

Animals were immunized with combinations of recombinant and native extracted proteins as per the scheme depicted in Table 1. An adjuvant control group with MPLA, DDA and LNP was used as a comparator against all test vaccine groups (10 animals per group). Each of the following combinations of antigens were formulated with MPLA+DDA or MPLA+LNP: nMOMP; nMOMP + PmpG; and nMOMP + PmpG + PmpD-p73 + PmpD-p82 (Table 1). A live Cm EB group was administered intraperitoneally as a positive control for protection against Chlamydia challenge (26, 27). Sera collected prior to primary immunization and on day 42 were tested in ELISAs for antibodies to nMOMP, *C. muridarum* EBs, PmpG, PmpD-p73 and PmpD-p82. Anti-nMOMP IgG titers were high for all vaccine groups with the MPLA/LNP adjuvanted groups (3, 5, 7) inducing significantly higher ($P < 0.001$) IgG titers than MPLA/DDA adjuvanted groups (2, 4, 6) (Figure 3A). The IgG1/IgG2c ratios for MPLA/LNP groups compared to MPLA/DDA groups were not statistically different, although the ratios indicate an overall balanced IgG1/IgG2c responses for most vaccinated groups with the exception of group 2 (nMOMP + MPLA+ DDA), group 6 (containing all antigens: nMOMP, PmpG, PmpD-p73, PmpD-p82+MPLA+DDA) and group 8 (live EB), which were lower, favoring IgG2c. However, comparisons of group 1, 2, 6 or 8 were not statistically different from each other (data not shown). Groups with ratio's < 1 indicate more IgG2c thus suggesting a bias towards Th1/IL-17 cytokine responses (Figure 3B). All vaccine groups had high IgG titers to *C. muridarum* EBs, with MPLA/LNP adjuvanted groups (groups 3 and 5) having statistically higher ELISA titers than MPLA/DDA adjuvanted groups (groups 2 and 4) (Figure 3C). Those groups containing PmpD-p73 and PmpD-p82 had high PmpD IgG titers, with the MPLA+LNP adjuvanted group (group 7) having approximately 1 to 3 logs higher titers than the MPLA+DDA adjuvanted group (group 6) (Figure 3D, 3E). Some cross reactivity to both PmpD-p73 and PmpD-p82 was observed for groups vaccinated with PmpG. Groups containing PmpG had high IgG titers to PmpG with the exception of group 6 (nMOMP/PmpG/PmpD p73/PmpD p82/MPLA/DDA) which had a wider of titers against the Pmp antigens tested. Interestingly, PmpG antigens formulated with LNP, group 5 and 7, had higher titers than those formulated with DDA, groups 4 and 6, although only group 4 and 5 were significantly different ($P < 0.0001$) (Figure 3F).

Immunization with native and recombinantly expressed proteins resulted in early clearance of C. muridarum following genital tract infection

Following immunization, animals were challenged with *C. muridarum* intravaginally (10 animals per group). Bacterial burden was assessed by swabbing the vaginal vault and ectocervix. Samples were then evaluated via qPCR as described previously (21). Data were plotted as Area Under the Curve (AUC) for individual mice and further analyzed by Wilcoxon Rank Sum Test (compared to MPLA / DDA / LNP). All vaccine test groups with either adjuvant MPLA + DDA or MPLA + LNP had reduced bacterial burden and early clearance over time when compare to the adjuvant MPLA / DDA / LNP control group (Figure 4). The reduction in bacterial shedding from the vagina for all groups was statistically significant ($P < 0.001$), with

Group 5 MOMP + PmpG + MPLA/LNP having a slightly lower statistical significance ($P = 0.003$). The EB positive control group was also protected from *Chlamydia* infection (Figure 4). Although the test vaccines appeared to decrease *Chlamydia* burden over the course of the infection, they failed to achieve the degree of protection provided by intraperitoneal administration of EBs. No statistical differences were observed between any of the vaccine groups (2–7). The EB positive control group was statistically lower than each of the vaccine test groups (2–7), p-values not shown.

Discussion

The development of an efficacious vaccine against *Chlamydia* is still an important unmet medical need. Since the first vaccine trials in humans more than 50 years ago, there have been valiant efforts to produce a vaccine against *Chlamydia*, to prevent both trachoma and genital tract infections. The early human vaccine trials produced valuable information regarding the lack of efficacy of formalin inactivated whole *Chlamydia* vaccines and the possible immunopathology that results from their use. Additionally, over the years, animal models have elucidated the protective immune responses necessary to clear both primary and secondary infections. There is hope that these animal models will inform us about the human disease and the findings will translate to protection in humans. The murine primary protective immune response has been found to be MHC-II restricted CD4+ T-cells with MHC-I restricted CD8+ T-cells playing a less critical role in protection (22–25). Antibodies will also be important in vaccine development as the protective role for antibodies has been demonstrated in relation to reinfection following a primary *C. muridarum* infection in mice (28–30). Cytokines of a Th1 profile such as IFN- γ and interleukin 12 (IL–12) are essential against *C. muridarum* infection in mice (31, 32). In addition, IL–17 mediated immunity has been shown to play a role in the control of intracellular pathogens, including *Chlamydia* (10, 15). However, there is also some evidence for a pathogenic role for IL–17 through enhanced recruitment of polymorphonuclear neutrophils and inflammatory monocytes in the UGT. The Th17-mediated inflammation has been associated tissue destruction and with intra-abdominal adhesions and increases in splenic weights in the mouse model (15–17).

In this study we compared subunit vaccines of native extracted MOMP either alone or in combination with other recombinantly expressed outer membrane MHC-II restricted proteins (PmpG, PmpD-p73 and PmpD-p82) to evaluate whether the addition of Pmp proteins would confer a greater reduction in bacterial shedding after challenge compared to nMOMP alone. In addition, we included various adjuvants to drive the cell mediated immune response toward Th1 and Th1/Th17 and to address the role of IL–17 in protection. The vaccines were adjuvanted with MPLA/DDA or MPLA/LNP and tested in C57Bl/6 mice. MPLA elicits a strong Th1 and balanced IgG1/IgG2 response when formulated with protein vaccines. LNPs when co-mixed with some sub-unit antigens result in strong B-cell responses, CD4+ and CD8+ T-cell responses (33). The B-cell responses are typically Th2-like (based on IgG2a:IgG1 ratio). The antigen specific CD4+ and CD8+ T-cell responses boosted by LNP are typically polyfunctional: IFN- γ +TNF- α +IL–2+ (33). DDA has been shown to increase Th1 responses yielding a mixed Th1:Th2 response and an increased IL–17+ response (34).

High systemic antibody titers were seen overall in this study. The LNP+MPLA adjuvanted groups had higher antibody titers when compared to MPLA+DDA adjuvanted group. Interestingly higher titers of antibodies in the LNP+MPLA groups did not translate to better clearance of *Chlamydia* following the intravaginal challenge. There can be several potential explanations for this observation, including higher amplitude of serum antibody titer may not reflect the antibody titers in the mucosal compartment thus not translating into improved protection from vaginal challenge. Furthermore, the functionality of the antibodies raised could be different for the two groups of adjuvanted formulations. We plan to evaluate functional mucosal responses in future studies.

In these studies, we observed a reduction in overall bacterial shedding in vaccine test groups that were statistically better than the adjuvant control group. We observed that an increased Th17+ response did not add to the protective immune responses nor did the addition of any of the Pmp family of antigens to a native MOMP vaccine elicit an increase in protection following intravaginal challenge. Our data also indicated that eliciting Th1 responses in the absence of Th17 responses were equally protective as MPLA+LNP formulations were comparable to MPLA+DDA formulations in terms of reducing the *Chlamydia* load after challenge. These data are consistent with publications that have shown that the protective immune response to *Chlamydia* infection appears to be correlated with a dominant Th1 and low Th17 T-cell response (26). Mice in the DDA formulated groups did not have significantly lower bacterial shedding after challenge as compared to the mice in the LNP adjuvanted groups (Group 2 vs Group 3 p-value = 0.68, Group 4 vs Group 5 p-value = 0.38, Groups 6 vs Group 7 p-value = 0.31, Figure 4). This suggests that the ability of the DDA-formulated vaccines to elicit an IL-17 response did not confer greater protection from shedding than IFN- γ , IL-2 and TNF- type Th1 response in this challenge model.

No significant differences were observed in reduction of pathology for any of the groups including the live EB immunized group in this study (data not shown). Due to similar pathology observed across all adjuvanted groups and EB vaccinated group, the role of IL-17 in inducing pathology could not be determined. Consistent to our observation, it has been reported that EBs immunized intranasally were more resistant to genital tract pathology than EBs immunized intraperitoneally in mice upon subsequent intravaginal *Chlamydia* challenge. This observation could be postulated to better mucosal immunity upon intranasal immunization compared to intraperitoneal vaccinations.(26). A good mucosal immune response may prevent *Chlamydia* ascension to the upper genital tract. Future studies could include for academic purposes, intranasal immunization with live EBs to test whether this immunization route would be more resistant to genital tract pathology consistent with the literature.

Our nMOMP immunized groups conferred protection from challenge similar to previous publications (6–8, 18). For those groups combining MOMP and either PmpD or PmpG or a combination of both PmpD and PmpG (PmpDG), we did not see an increased reduction of bacterial shedding compared to nMOMP alone after intravaginal challenge. This data was in contrast to previous reports that showed the combination of Pmp E, F, G, H + MOMP reduced shedding over MOMP alone, PmpG alone, or the combination of Pmps (11). However, it should be noted that there are marked differences between the studies performed herein and those reported previously. Differences include the combination of Pmps,

protein purification methods, study designs (antigen dose, adjuvants), assessment following challenge (sample collection, *Chlamydia* shedding assay), all of which may theoretically result in the lack of increased protection for the combination vaccine image. The Pmps in our study were selected based on recombinant protein expression and availability; a future study would need to include additional recombinantly expressed Pmps such as E, F, and H. In addition to including more Pmps in a future study, a stricter alignment using the same protein purification methods, the use of same adjuvants, and assessment following challenge is warranted for a closer comparison between the data sets. Further, a direct comparison of the reagents and antigens generated from the previous studies with our reagents and vaccine combinations in a side by side comparison would be beneficial to elucidate why the results were not similar.

Conclusions

In conclusion, we found that *Chlamydia* vaccines based on nMOMP provided modest protection against intravaginal challenge with *Chlamydia muridarum*. The efficacy of these vaccines could not be improved either by the incorporation of additional Pmp antigens, or by formulating the nMOMP antigen with adjuvants that either increased circulating anti-nMOMP IgG, or by eliciting a Th17+ response to nMOMP. An efficacious vaccine will require finding the optimal combination of antigens with several targets included and an adjuvant/immunization route combination that results in multifunctional CD4+ T cells and humoral responses elicited systemically and in the mucosal compartments.

Abbreviations

IFU, inclusion forming unit; EB, elementary body; MOMP, Major Outer Membrane Protein; PmpG, polymorphic membrane protein G; PmpD polymorphic membrane protein D; intracellular cytokine staining (ICS), IL-17, Interlukin-17, MPLA Monophosphoryl lipid A, DDA dimethyldioctadecylammonium bromide, upper genital tract (UGT)

Declarations

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

All authors are employee of Merck & Co., Inc., West Point, PA, USA

Ethics Approval and Consent to Participate

All animal experiments were approved by the Merck & Co., Inc., Kenilworth, NJ, USA, Institutional Animal Care and Use Committee (IACUC). All procedures were performed in accordance with our institution's

IACUC guidelines in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. Mice were housed in large mouse containers (n = 10 mice/box) with micro isolator lids, and the rooms were maintained with controlled humidity and temperature, and 12-hour light-dark cycles. All containers had nestlets and animals were provided standard chow (Purina 5001 rodent diet) and water ad libitum. The physical condition of the animals was monitored daily (7 days a week), and any health changes were noted.

At the end of the study, animals were euthanized using CO₂ inhalation (10% - 30% CO₂ in the air mixture inhaled per minute). No animals were found to be moribund throughout the study.

Authors' Contribution

MB is the primary author, and performed recombinant protein cloning and expression, and performed additional data analysis for ELISAs and qPCR evaluations. RK conducted the animal studies and performed ELISAs and data analysis of the ELISAs. KC ran the FAQ assays and performed the data analyses and authoring of the related sections for FAQ analysis. BL purified both recombinant proteins and native Momp proteins. JX, purified Chlamydia and ran ELISAs and performed data analysis for ELISAs. DN performed amino acid sequence analysis; ST ran qPCR testing and associated data analysis. AE, KV, and JS were pivotal in study design and conception. All authors have approved the manuscript.

Funding

Funding for this research was provided by Merck & CO., Inc., Kenilworth, NJ, USA.

Acknowledgments: The authors would like to thank Gokul Swaminathan for his critical analysis of the paper and MRL West Point Lab Animal Resources for their assistance with the animal studies.

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Figures

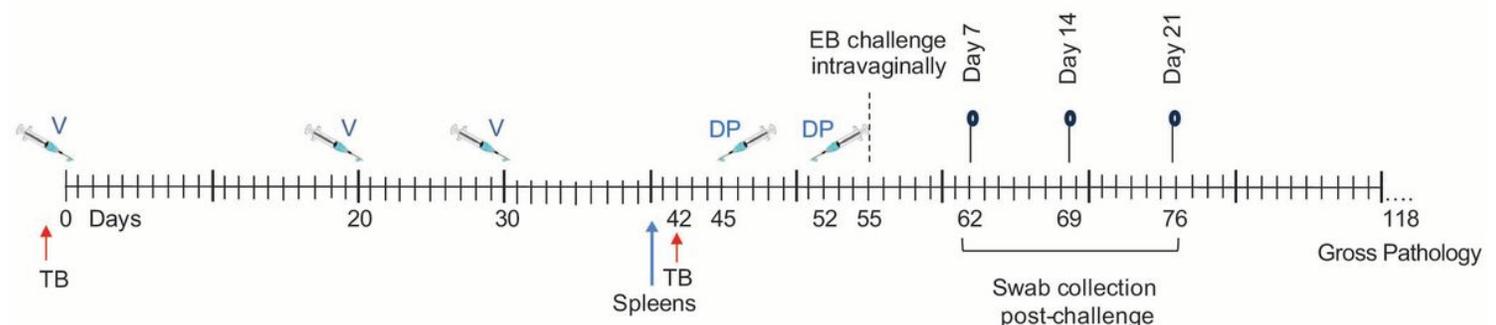


Figure 1

Study Design Schedule of vaccination, collection of spleens, Chlamydia muridarum challenge, cervicovaginal swabbing, and gross pathology in female C57BL/6 mice. V, vaccination; DP, depo-provera treatment; TB, tail bleeds; Splensens, harvested for ICS.

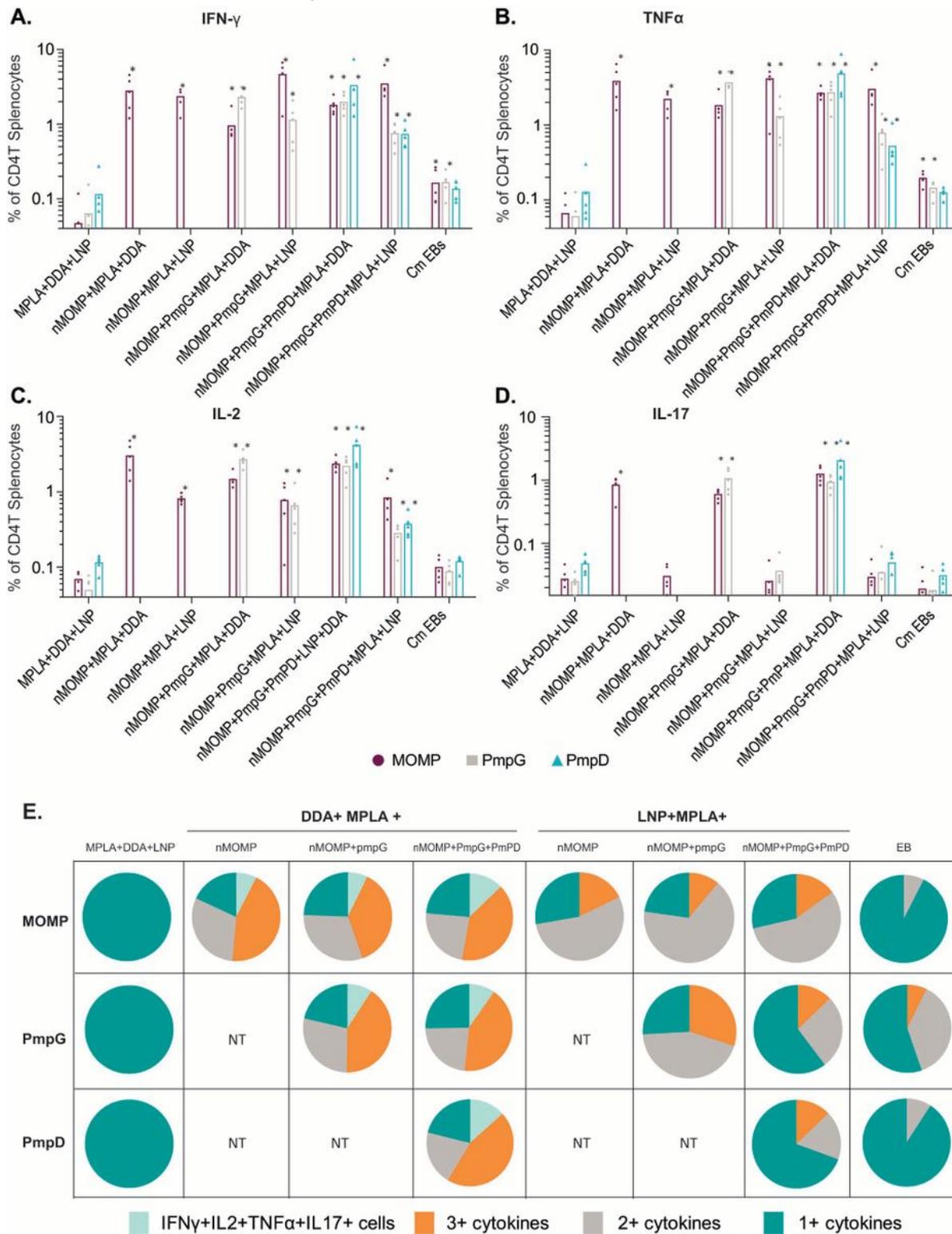


Figure 2

FACS Antigen specific cytokine responses after immunization with Chlamydia proteins formulated with adjuvants. Splenocytes from the adjuvant control group, vaccine immunized groups and Cm EB

vaccinated group were stimulated with 15mer peptide pools corresponding to the vaccine antigens for each group (MOMP, PmpG, PmpD). Bar charts represent the percent of CD4 T cells producing IFN- γ (2A), TNF- α (2B), IL-2 (2C) or IL-17 (2D) as evaluated by multiparameter flow cytometry. Individual points within each open bar graph represent data from an individual mouse, and the height of the open bar depicts the mean response for the group. * depicts a significant difference ($p < 0.05$) in response as compared to the adjuvant only group (MPLA+DDA+LNP) in a two tailed paired t test. NT, not tested.

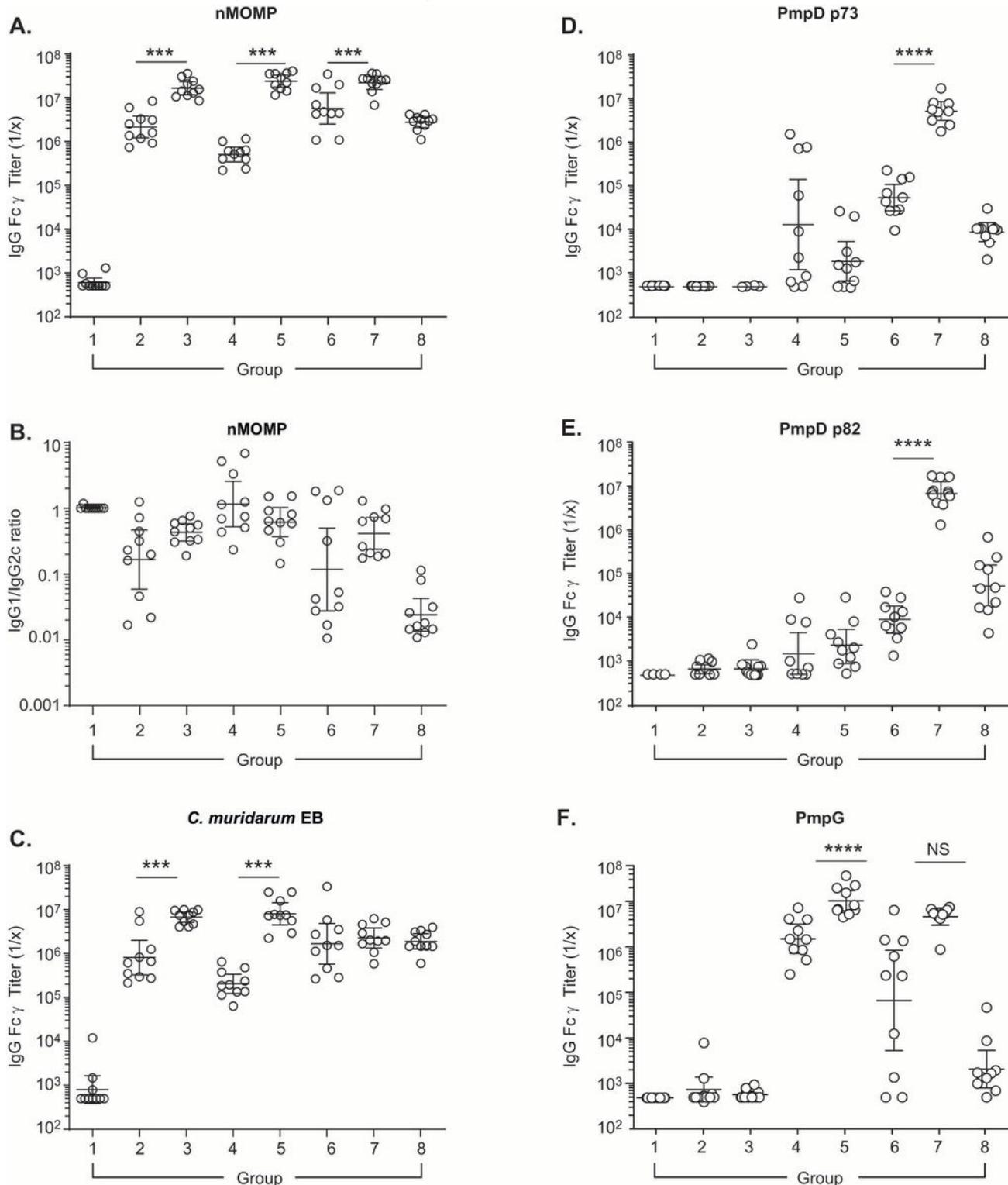


Figure 3

ELISA IgG antibody titers to nMOMP, EB, PmpG, and PmpD as determined by ELISA. 3A. Total IgG titers to nMOMP. 3B. IgG1/IgG2c ratio of anti-nMOMP titers. 3C. Total IgG titers to *C. muridarum* EBs 3D. Total IgG titers to PmpD p73 3E. Total IgG titers to PmpD p82, 3F. Total IgG titers to PmpG. Grp 1) MPLA+DDA+LNP, Grp 2) Cm nMOMP+MPLA+DDA, Grp 3) Cm nMOMP+MPLA+LNP, Grp 4) Cm nMOMP+PmpG+MPLA+DDA, Grp 5) Cm nMOMP+PmpG+MPLA+LNP, Grp 6) Cm nMOMP+PmpG+PmpD p73+PmpD p82+MPLA+DDA, Grp 7) Cm nMOMP+PmpG+PmpD p73+PmpD p82+MPLA+LNP, Grp 8) *C. muridarum* EBs. Error bars are geometric means with 95% confidence intervals. Transformed data analyzed by One-way ANOVA with Dunnett post test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, NS not significant

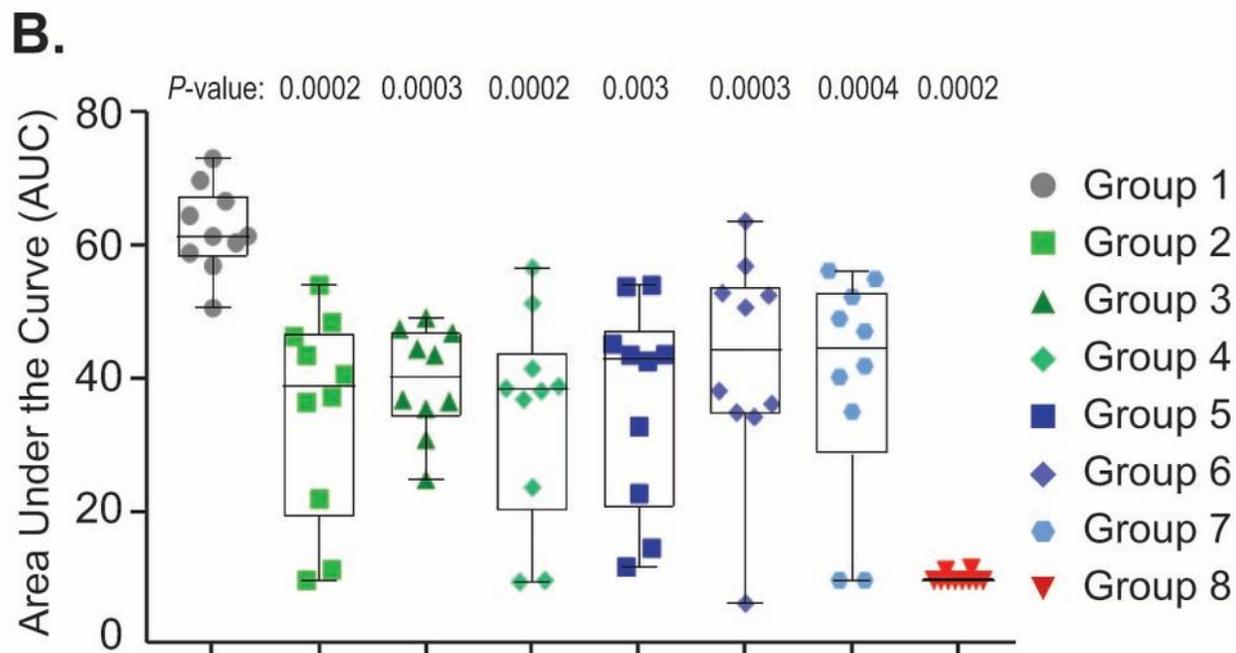
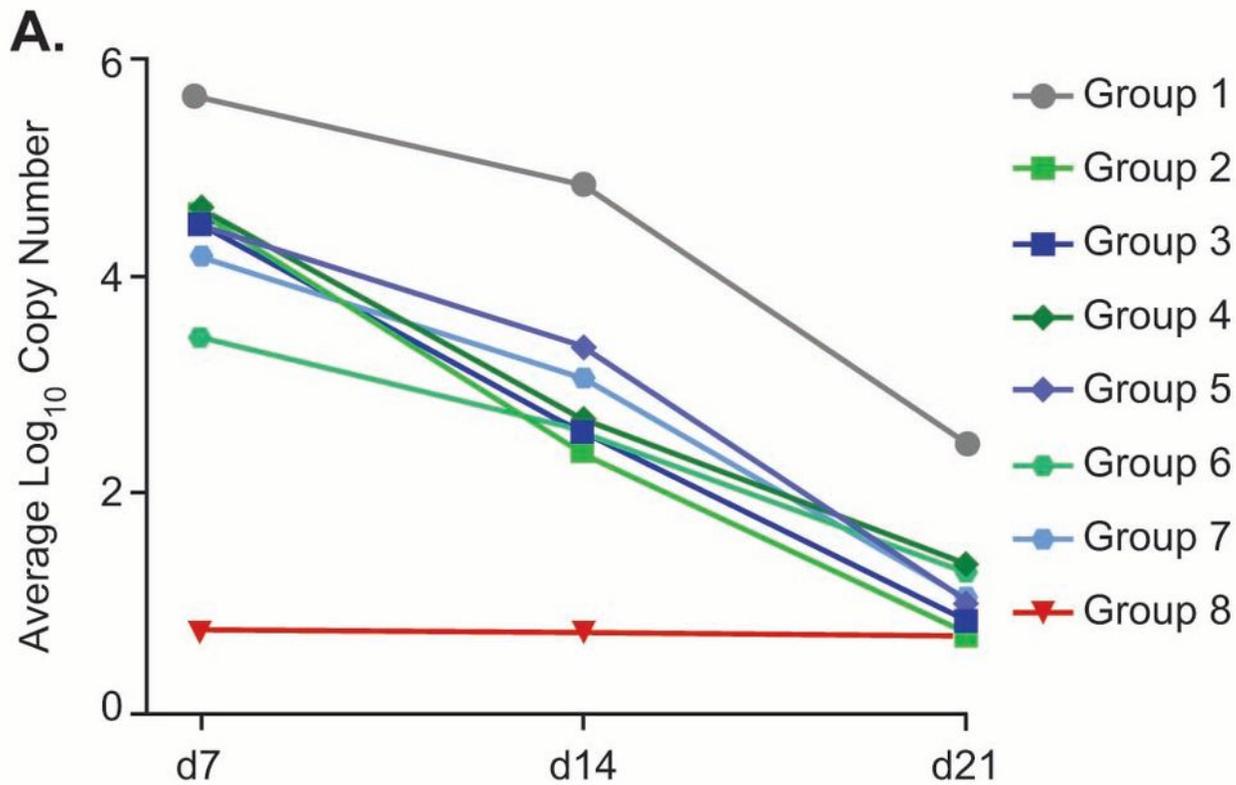


Figure 4

Chlamydia muridarum challenge in mice. 4A.) Average copy numbers from real-time qPCR for each vaccine test group were plotted on Log₁₀ scale over days post challenge. 4B.) Area under the curve (AUC) values for individual mice were calculated and plotted and further analyzed by Wilcoxon Rank Sum Test to determine the p-values for each group (compared to MPLA/DDA/LNP, Grp 1) MPLA+DDA+LNP, Grp 2) Cm nMOMP+MPLA+DDA, Grp 3) Cm nMOMP+MPLA+LNP, Grp 4) Cm

nMOMP+PmpG+MPLA+DDA, Grp 5) Cm nMOMP+PmpG+MPLA+LNP, Grp 6) Cm nMOMP+PmpG+PmpD p73+PmpD p82+MPLA+DDA, Grp 7) Cm nMOMP+PmpG+PmpD p73+PmpD p82+MPLA+LNP, Grp 8) C. muridarum EBs.

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