Increased Immunoaccessibility of MOMP Epitopes in a Vaccine Formulated with Amphipols May Account for the Very Robust Protection Elicited against a Vaginal Challenge with Chlamydia muridarum

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*J Immunol* 2014; 192:5201-5213; Prepublished online 28 April 2014; doi: 10.4049/jimmunol.1303392
http://www.jimmunol.org/content/192/11/5201

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*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Increased Immunoaccessibility of MOMP Epitopes in a Vaccine Formulated with Amphipols May Account for the Very Robust Protection Elicited against a Vaginal Challenge with *Chlamydia muridarum*

Delia F. Tifrea,* Sukumar Pal,* Jean-Luc Popot,† Melanie J. Cocco,‡ and Luis M. de la Maza* 

There is a need to implement a vaccine to protect against *Chlamydia trachomatis* infections. To test a new vaccine, mice were immunized with the *Chlamydia muridarum* native major outer membrane protein (nMOMP) solubilized with either amphipol A8-35 or the detergent Z3-14. OVA was used as a negative control, and mice were inoculated intranasally with *C. muridarum* as positive controls. Animals vaccinated with nMOMP mounted strong *Chlamydia*-specific humoral and cell-mediated immune responses. Mice vaccinated with nMOMP/A8-35 had a higher ratio of Abs to denatured elementary bodies (EB) over live EB, recognized more synthetic MOMP peptides and had higher neutralizing titers than sera from mice immunized with nMOMP/Z3-14. T cell lymphoproliferative responses and levels of IFN-γ were also higher in mice vaccinated with nMOMP/A8-35 than with nMOMP/Z3-14. Following immunization, animals were challenged intravaginally with *C. muridarum*. On the basis of the number of mice with positive vaginal cultures, length of vaginal shedding, total number of positive vaginal cultures, and number of *Chlamydia* inclusion forming units recovered, nMOMP/A8-35 elicited a more robust protection than nMOMP/Z3-14. By depleting T cells with Abs, we determined that CD4+ and not CD8+ T cells mediated the protection elicited by nMOMP/A8-35. Mice were subsequently mated, and based on the number of pregnant mice and number of embryos, animals that were vaccinated with nMOMP/A8-35 or nMOMP/Z3-14 had fertility rates equivalent to the positive control group immunized with live EB and the fertility controls. In conclusion, increased accessibility of epitopes in the nMOMP/A8-35 preparation may account for the very robust protection against infection and disease elicited by this vaccine. *The Journal of Immunology*, 2014, 192: 5201–5213.

*Chlamydia trachomatis* is worldwide the leading cause of bacterial sexually transmitted diseases and also can produce ocular, gastrointestinal, and respiratory infections (1, 2). Annually, up to 4–5 million new genital *C. trachomatis* infections are reported in the United States (1, 3). Although effective antimicrobial therapy is available, >50% of the chlamydial infections are asymptomatic, and even in symptomatic cases, treatment failures can occur (4). Furthermore, countries that have established screening programs, followed by antibiotic therapy, have observed an increase in the prevalence of the infection (5). This increase is thought to be because of a block in the development of natural immunity as a result of the antibiotic therapy (5, 6). Therefore, the implementation of a vaccine appears to be the best approach to control and eradicate these diseases (7–10).

In the 1960s, various investigators tested live and inactivated whole-organism vaccines in humans and nonhuman primates to protect against trachoma (2, 11–13). Several vaccine formulations were reported to be effective. However, the protection was found to be short-lived and serovar or serogroup specific. Furthermore, some immunized individuals suffered a hypersensitivity reaction upon reexposure to *Chlamydia* (2, 13–15). Although the cause of this hypersensitivity reaction is still under investigation, it is thought to be secondary to exposure to one of the antigenic components present in the whole bacterium (16). Thus, there is a need to formulate a subunit vaccine.

When the sequence of the *C. trachomatis* major outer membrane protein (MOMP) was analyzed, it was found to have regions of DNA unique to each serovar (17, 18). Therefore, the likelihood that the protection elicited by the trachoma vaccine was due to MOMP was considered (7, 8, 19). Unfortunately, attempts to elicit protection in several animal models using rMOMP, MOMP peptides, and DNA MOMP–based vaccines yielded disappointing results (20–22). The possibility that the native conformation of MOMP, or posttranslational modification of the protein, was necessary for protection instigated the search for preparations of the native MOMP. Using detergents, a trimeric form of MOMP, considered to correspond to its native structure, was isolated. Mice...
vaccinated with this preparation mounted a strong immune response that was protective against genital and respiratory challenges (23–27). Subsequently, the same type of preparation was found to elicit protection in nonhuman primates against an ocular infection (28). Detergents however, can have toxic effects at high concentrations and tend to destabilize membrane proteins accelerating their denaturation (29–33). Thus, detergents are not considered ideal vehicles to formulate vaccines.

In the 1990s, Tribet et al. (34) developed amphiphatic polymers, termed amphipols (APols), designed to keep membrane proteins soluble in water in the absence of detergent. This group of investigators showed that several integral membrane proteins, including Escherichia coli OmpF, a protein similar in structure to MOMP, were kept soluble in their native conformation by APols (35). On the basis of these findings, the Chlamydia nMOMP was extracted with detergents and transferred to APols. Immunoization of mice with this preparation was found to elicit a more robust protection against an intranasal chlamydial challenge than the detergent-based formulation (31). In this study, to assess the feasibility of eliciting protection against a vaginal challenge with Chlamydia and to characterize the immune mechanisms involved in protection, we tested in parallel nMOMP preparations formulated in detergents and in APols. Our results show that nMOMP formulated with APols induces very robust protection against a vaginal infection and preserves fertility. The protection is dependent on CD4+ T cells and may result from the increased accessibility to the immune system of epitopes in the nMOMP/A8-35 formulation when compared with the nMOMP/Z3-14 preparation.

Materials and Methods

Stocks of Chlamydia muridarum

The C. muridarum strain Nigg II (previously called C. trachomatis mouse pneumoniais biovar, strain Nig II; obtained from the American Type Culture Collection, Manassas, VA) was grown in McCoy cells (36). Elementary bodies (C. muridarum–EBs) were purified and stored in SPG (0.2 M sucrose, 20 mM sodium phosphate [pH 7.2], and 5 mM glutamic acid) as described previously (37).

Purification and preparation of C. muridarum nMOMP

The nMOMP was extracted and purified as described previously (25, 26). Briefly, C. muridarum was grown in McCoy cells, harvested, and washed with PBS. Following digestion of the pellet with DNase, the nMOMP was extracted by incubating twice with CHAPS (Anatrace, Maumee, OH) and once with Z3-14 ([n-tetradecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate; Anzergent 3-14; Anatrace]). The supernatant was transferred to a hydroxyapatite column was incubated at room temperature for 2 h with APol A8-35 ([n-tetradecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate; Anzergent 3-14; Anatrace]. The supernatant was transferred to a hydroxyapatite column, and the MOMP trimer was eluted using a phosphate buffer gradient (37).

The purity of the nMOMP preparation was assessed by several methods. Using the limulus amebocyte assay (BioWhittaker, Walkersville, MD), the nMOMP was found to have <0.05 EU LPS/μg protein (24). Mass spectrometry analyses and N-terminal sequencing of nMOMP both revealed a purity of >99% (38, 39). The apparent molecular mass was determined by SDS-PAGE (40).

To prepare the nMOMP/Z3-14 for immunization the protein was concentrated and fixed with 2% glutaraldehyde (Sigma-Aldrich, St. Louis, MO) at room temperature for 2 min as described previously. Glycine (Bio-Rad Laboratories, Hercules, CA) was added to stop the reaction. The nMOMP/Z3-14 was dialyzed against PBS (pH 7.4) with 0.05% Z3-14 before immunization. To formulate the nMOMP/A8-35, the protein from the hydroxyapatite column was incubated at room temperature for 2 h with APol A8-35 ([n-tetradecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate; Anzergent 3-14; Anatrace] at a weight ratio of 2/1 (31). To remove the Z3-14, rehydrated BioBeads SM-2 Adsorbent (Bio-Rad, Hercules, CA) were added at a weight ratio of 1.25 × 103 (Z3-14/Biobeads), and the mixture incubated at 4°C for 16 h when the beads were removed by centrifugation.

Characterization of the nMOMP/A8-35 and nMOMP/Z3-14 preparations by nuclear magnetic resonance

To label the nMOMP with 15N, following infection with C. muridarum, the McCoy monolayers where cultured with BioExpress 2000 (U-15N) insect cell media (Cambridge Isotopes Laboratories, Andover, MA) supplemented with 1 mg/ml glucose, 50 μg/ml gentamicin sulfate, and 1 μg/ml cycloheximide. The nMOMP was extracted as described above, and the trimeric structure was confirmed by SDS-PAGE. The nuclear magnetic resonance (NMR) samples were prepared in a volume of 300 μl with 10% D2O, 100 mM sodium phosphate (pH 7.4), and 300 μM nMOMP in 25 mM dodecylphosphopholine (DPC; Anatrace and 300 μM nMOMP with 24 mg/ml APoI A8-35, as described above). The NMR 15N heteronuclear single quantum coherence (HSQC) data were collected on a Varian Inova 800-MHz spectrometer (160 scans, 1024 points, 48 increments). Data were processed using an NMR pipeline (41).

Immunization protocols

To elicit protection before the mice reached sexual maturity, 3-wk-old female BALB/c (H-2b) mice (Charles River Laboratories, Wilmington, MA) were vaccinated with the nMOMP, or OVA (Sigma-Aldrich), twice, 2 wk apart, by the colonic route (10 μg protein/mouse/imunization), followed by two times intranasally (31). In this formulation, plus the s.c. (3 μg protein/mouse/imunization) routes. Mucosal, followed by systemic immunization, has been found to be the most effective vaccination schedule for inducing protection against a chlamydial genital challenge (42). CpG-1826 (10 μg/mouse/dose; 5’-TCCATGACGTTCCTAGCGTT-3‘; Trilink Biotechnologies, San Diego, CA) and Montanide ISA 720 VG (Seppic, Fairfield, NJ) were used as adjuvants (24, 42, 43). Montanide was only applied by systemic routes. For colonic route immunization, mice were kept without food overnight, and the vaccinations were performed using a 4.5-French gauge dosing catheter (Harvard Apparatus, Holliston, MA). OVA was solubilized in PBS, mixed with Z3-14 or APols like MOMP and used as a negative control. Another negative control group was inoculated intrathoracically (i.n.) with MEM without serum (MEM-0). Positive control mice were immunized i.n. once with 1 × 104 inclusion forming units (IFU) of C. muridarum (45). All experiments were repeated twice. The animal protocols were approved by the University of California, Irvine, Animal Care and Use Committee.

Immunoassays

Blood was collected from the periorbital region, and C. muridarum–specific Ab titers in sera were determined by an ELISA as described previously (23, 45). Briefly, multwell plates (Corning Glass Works, Corning, NY) were coated overnight with 1 μg purified native or boiled (for 30 min in the presence of 1 μg/ml 2-ME) C. muridarum–EB per well, and serial dilutions of serum were added. The Ag–Ab reactions were detected with HRP-conjugated goat anti-mouse Abs. The following Abs were used: IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD), IgG1, IgG2a, and IgA (Southern Biotechnology Associates, Birmingham, AL). The substrate 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic) acid was used for color development. The plates were scanned at 405 nm in an ELISA reader (Labsystem Multiscan, Helsinki, Finland). Titers were calculated using as a background the OD of preimmunization sera ± 2 SD and reported as geometric mean titer (GMT).

To detect Abs elicited by vaccination to B cell–specific linear epitopes, overlapping 25-mer corresponding to the mature C. muridarum MOMP amino acid sequence were chemically synthesized (SynBioSci, Liverton, CA) (46). Peptide 25 overlaps the N and C terminus of MOMP. The peptides were adsorbed onto high binding affinity ELISA plates (10 μg/ml; 100 μl/well of a 96-well plate), and the Ab binding was determined in triplicates as described above using a 1:200 dilution of serum and a 1/10,000 dilution of goat anti-mouse IgG (47).

In vitro neutralization assays were performed as published previously (48). A total of 1 × 104 IFU C. muridarum were added to serial dilutions of mouse sera made with Ca2+- and Mg2+-free PBS (pH 7.2) and supplemented with 5% guinea pig serum. After incubation for 45 min at 37°C, the mixture was incubated by centrifugation into HeLa-229 cells grown on shell vials. After 30 h of incubation at 37°C, the monolayers were fixed and stained with a pool of mAbs to MOMP, the 60-kDa cysteine-rich protein, the 150-kDa putative outer membrane protein, and the LPS, prepared in our laboratory (45). The titer of a sample was the dilution that yielded 50% neutralization relative to the negative control serum from MEM-0–immunized mice.

A T cell lymphoproliferative assay (LPA) was performed using spleenocytes as previously described (45). In short, splenic T cells were collected and UV-inactivated C. muridarum–EB, or nMOMP/A8-35, were added at concentrations of 105–106 CB/μl, or 20 μg nMOMP/A8-35, to 1 APC, which were prepared by irradiating spleenocytes with 3000 rad [37Cs]. Negative control wells received medium (RPMI 1640 medium + 10% FBS) alone and positive controls wells Con A (5 μg/ml). At 96 h of incubation,
cell proliferation was measured by addition of 1 μCi [methyl-3H]thymidine (47 Ci/mmol; Amersham Biosciences, Arlington Heights, IL)well. The mean count was obtained from triplicate cultures. The LPA also was used to assess the toxicity of the nMOMP/Z3-14 and nMOMP/A8-35 preparations using splenocytes from naive mice and Con A as a nonspecific stimulant.

Levels of IFN-γ and IL-4 were determined using commercial kits (BD Pharmingen, San Diego, CA) in supernatants from splenic T cells stimulated as described above (31).

**T cell depletion**

Anti-CD4 rat IgG2b (78,15. mCD4) and anti-CD8 rat IgG2a (53-6.72 mCD8) Abs (BioXCell, West Lebanon, NH) were administered i.p. Animals received 500 μg/mouse of each Ab, at days (−5) and (−1) before the challenge, followed by 250 μg/mouse of each Ab, twice a week for 5 wk after challenge (49, 50).

Flow cytometry analysis was used to verify in vivo T cell depletion before the genital challenge. Four mice were included in each monoclonal-treated group, and four animals, inoculated with PBS, were used as controls. Mice were euthanized, and whole spleen cell populations (2 × 10⁶ total cells; 95% viable as determined by trypan blue exclusion) were stained for 45 min at 4°C with anti–CD4-PE, anti–CD8a-PerCP, or fluorescent-conjugated rat (IgG2a and IgG2b) isotype control Abs (5 μg/10⁶ splenocytes; BD Biosciences, San Diego, CA). Cells were washed twice, analyzed by flow cytometry (BD FACSCalibur) for at least 10,000 events/sample and gated for lymphocytes. Dead cells and monocytes were excluded using forward and side scattering gating.

**Intravaginal challenge**

Mice were treated s.c. four days before the challenge with 1 mg/mouse of medroxy progesterone acetate (Greenstone, Peapack, NJ) (51). At 4 wk after the last immunization, mice were inoculated intravaginally (i.vag.), with 10⁴ IFU of C. muridarum in 20 μl SPG (45, 52). To confirm that all mice were on diestrus, the vaginal cytology was checked before the challenge.

**Genital cultures**

Vaginal swabs were cultured twice weekly for the first 2 wk and then at 7-d interval for an additional 4 wk following the genital challenge (45). HeLa-229 cells grown in 48-well tissue culture plates were inoculated with 10-fold dilutions of the vaginal swabs and incubated for 30 h at 37°C. The monolayers were fixed with methanol, and chlamydial inclusions were stained using a pool of mAb described above. The limit of detection was 2 IFU/culture.

**Fertility studies**

At 6 wk following the challenge, female mice were housed with proven breeder male mouse for 18 d and then repeated once if necessary (45). Fertility is defined as at least one embryo per mouse.

**Statistics**

The two-tailed unpaired Student t test, and the Fisher’s exact test were used for statistical analysis with the program SigmaStat version 3.5. Differences were considered significant for p values < 0.05.

**Results**

**Ab responses in serum following immunization**

BALB/c mice were vaccinated with nMOMP/A8-35 or nMOMP/Z3-14 using CpG and Montanide as adjuvants and the negative control groups with OVA instead of nMOMP. As a positive control, mice were immunized with live C. muridarum-EB, and as a non-immunization control, animals received MEM-0. Serum samples were collected the day before the i.vag. challenge with C. muridarum. As shown in Table I, animals vaccinated with nMOMP/A8-35 had similar C. muridarum–specific IgG GMT, using native EB as the Ag, when compared with mice immunized with nMOMP/Z3-14 (51, 200 versus 62,413; p > 0.05). The control group immunized with 10⁴ IFU C. muridarum-EB had an IgG GMT of 8,063. Mice immunized with OVA/Z3-14, or OVA/A8-35, had IgG Ab titers below the limit of detection. To determine whether immunization with the different vaccine preparations
elicted Abs to epitopes not accessible in the native EB, IgG levels were also determined using boiled EB. Higher increases in Abs to epitopes not accessible in the native EB, IgG levels were also determined using boiled EB. Higher increases in Ab levels to boiled EB were observed in mice vaccinated with nMOMP/A8-35 (304,332/51,200; ratio = 6.86), or nMOMP/Z3-14 (226,118/62,413; ratio = 4.00), than in animals immunized with EB (10,159/8,063; ratio = 1.33) (226,118/62,413; ratio = 4.00), than in animals immunized with EB (10,159/8,063; ratio = 1.33). To assess whether a Th1- or a Th2-type response was induced, the titers of IgG1 and IgG2a were measured (Table I). In the two groups of mice immunized with nMOMP, the ratios of IgG2a to IgG1 were 16:1 for the nMOMP/Z3-14 and 10:1 for nMOMP/A8-35 groups, indicating a strong Th1-biased response. This ratio was 10:1 for the C. muridarum-EB control group. The Th1-biased response was also supported by the IFN-γ and IL-4 data (Table II).

The IgG GMT in serum was low in mice immunized with nMOMP/Z3-14 (336) or nMOMP/A8-35 (238) and not statistically different from each other. In the positive control group inoculated with C. muridarum-EB, the IgG GMT was 1131. Higher titers of neutralizing Abs were found in sera from mice vaccinated with nMOMP/A8-35, or C. muridarum-EB (1250), when compared with nMOMP/Z3-14 (250; p < 0.05).

The IgG GMT in vaginal washes were not statistically different between the nMOMP/A8-35 (381; range 160–1,280), the nMOMP/Z3-14 (160; range 40–320)– and the C. muridarum-EB (25; range 10–80)–immunized groups. Low levels of IgA were detected in the vaginal washes of mice immunized with both nMOMP/A8-35 (20; range 10–40) and the nMOMP/Z3-14 (16; range 10–20) preparations as well as with C. muridarum-EB (32; range 20–40).

Mapping of MOMP B cell epitopes
To determine the B cell epitopes recognized by Abs elicited by immunization, ELISA plates coated with 25-mer MOMP overlapping peptides were probed with serum samples collected the day before the i.n. challenge (Fig. 1). Sera from mice immunized with live EB bound exclusively to peptides corresponding to the variable domains (VD), preferentially VD1 (p5, p6), VD2 (p10), and VD4 (p20). Sera from mice vaccinated with nMOMP/A8-35, or nMOMP/Z3-14, also reacted with all four VD and in addition bound to peptides that included domains of VD1 (p4) and VD2 (p9). CD2 (p7) and CD5 (p23, p24) were also recognized by Abs from these two groups of nMOMP-immunized animals. Only sera from mice vaccinated with nMOMP/A8-35 bound to p14 (CD3), p19 (that includes regions of CD4 and VD4), and p21 (that overlaps VD4 and CD5). In contrast, p22 in CD5 was only recognized by Abs from mice immunized with nMOMP/Z3-14. Ten peptides (p1, p2, and p3 in CD1; p8 in CD2; p11, p12, and p13 in CD3; and p16, p17, and p18 in CD4) were not recognized by any sera from the three groups of immunized mice.

Evaluation of the toxicity of the nMOMP/A8-35 and nMOMP/Z3-14 preparations
To assess the potential cell toxicity of the nMOMP/A8-35 and nMOMP/Z3-14 preparations, splenic T cells from naive mice were stimulated non-specifically in vitro with Con A. Although T cells stimulated with Con A in the presence of nMOMP/A8-35 proliferated to levels similar to those stimulated only with Con A (24,627 ± 9,909 versus 67,226 ± 25,971 Δcpm; p > 0.05), cells stimulated with Con A in the presence of nMOMP/Z3-14 (130 ± 107 Δcpm) did not, indicative of the toxicity of the detergent (Fig. 2). Therefore, the T cell responses in immunized mice were evaluated using only EB and nMOMP/A8-35 as Ags.

Table II. T cell–proliferative responses of immunized mice from the day before the i.vag. challenge with C. muridarum

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Stimulated with C. muridarum-EB</th>
<th>Stimulated with nMOMP/A8-35</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T Cell Proliferation(^{ab}) (Δcpm) (\times 10^3)</td>
<td>SI(^b)</td>
</tr>
<tr>
<td>nMOMP/Z3-14</td>
<td>4.67 ± 3.46(^{cd})</td>
<td>20.13 ± 10.60(^{cd})</td>
</tr>
<tr>
<td>nMOMP/A8-35</td>
<td>14.29 ± 1.59(^{cd})</td>
<td>31.10 ± 20.30(^{cd})</td>
</tr>
<tr>
<td>OVA/Z3-14</td>
<td>0.49 ± 0.12</td>
<td>2.10 ± 0.30</td>
</tr>
<tr>
<td>OVA/A8-35</td>
<td>0.42 ± 0.17</td>
<td>2.40 ± 0.90</td>
</tr>
<tr>
<td>C. muridarum-EB</td>
<td>14.57 ± 5.15</td>
<td>47.90 ± 17.83</td>
</tr>
</tbody>
</table>

\(^{a}\)The ratio of UV-inactivated C. muridarum-EB to APC was 1:1.

\(^{b}\)The values are means ± 1 SD of four different cultures.

\(^{c}\)The values are means ± 1 SD of four different cultures.

\(^{d}\)Significant higher levels of IFN-\(\gamma\) were found in the groups vaccinated with the nMOMP/A8-35 (20.09 ± 3.63 ng/ml) and nMOMP/Z3-14 (15.43 ± 8.74 ng/ml) preparations when compared with their respective OVA-immunized groups (1.10 ± 1.24 and 1.12 ± 1.10; p < 0.05). These levels, however, were significantly lower than those found in the animals immunized with C. muridarum-EB (33.59 ± 0.91 ng/ml; p < 0.05) but not different between them (p = 0.062). IL-4 was only detected in the nMOMP/A8-35–immunized (7.56 ± 3.19 pg/ml) and C. muridarum-EB–immunized (5.36 ± 1.10 pg/ml) groups.
We prepared a fully 15N-labeled nMOMP sample for NMR studies and collected a one-dimensional proton NMR spectrum in Z3-14 or A8-35. Using a band-selective pulse centered on the aromatic region, our experiment was designed to observe aromatic and amide signals only without interference of the large detergent signals in the aliphatic range (53). Unfortunately, NMR signals of the detergent overlapped with the nMOMP signals.

**Figure 1.** Binding of serum Abs to synthetic *C. muridarum* MOMP peptides. Serum samples from mice immunized with nMOMP/A8-35, nMOMP/Z3-14, or EB, were collected the day before the i.vag. challenge and their reactivities to 25-mer peptides corresponding to the *C. muridarum* mature MOMP were analyzed by ELISA.

Characterization of nMOMP/A8-35 and nMOMP/detergent by NMR

We prepared a fully 15N-labeled nMOMP sample for NMR studies and collected a one-dimensional proton NMR spectrum in Z3-14 or A8-35. Using a band-selective pulse centered on the aromatic region, our experiment was designed to observe aromatic and amide signals only without interference of the large detergent signals in the aliphatic range (53). Unfortunately, NMR signals of
nMOMP were too broad to detect in the Z3-14 environment (data not shown). This was not unexpected because the average Z3-14 micelle particle alone is 30 kDa, and when added to a membrane protein, this contributes significantly to the total size of the particle. In fact, Z3-14 is rarely used in NMR studies because large particles give broad lines (often too broad to detect). DPC micelles have an average mass of 17 kDa and are considered a superior membrane substitute because protein–DPC particles are smaller and tumble faster. In addition, the phosphocholine head group of DPC is a natural feature of cellular membranes. Because we could not get valid NMR data in Z3-14, we used DPC. The Z3-14 sample was split and transferred into DPC or A8-35. NMR spectra of these samples are shown in Fig. 3. Trp signals appear in a distinct region of the spectrum, and therefore, their solvent accessibility can be assessed. Also, Trp side chains allowed to rotate freely in solution give the sharpest NMR signals. nMOMP contains eight Trp residues; seven of these are positioned in proximity to the membrane surface depicted in a model of MOMP (Fig. 3A). Fig. 3B and 3C shows regions where Trp indole protons typically resonate. In the nMOMP/DPC spectrum, we find only one weak signal. The lack of Trp signals in Fig. 3B is consistent with these side chains being restricted and occluded by detergents (DPC). In contrast, we find five strong Trp signals in the spectrum of nMOMP/A8-35 (Fig. 3C). Because the nMOMP/A8-35 particle size is still large, we can only detect signals corresponding to flexible, solvent accessible regions. The presence of Trp signals in the nMOMP/A8-35 NMR spectrum indicates these groups have increased exposure compared with Trp rings in the nMOMP/DPC sample.

Recovery of C. muridarum from vaginal cultures

The course of the infection was assessed, using vaginal cultures, twice a week for the first 2 wk postchallenge and once a week for four additional weeks (Figs. 4A, 5, Table III). The numbers of mice vaccinated with nMOMP/A8-35, or nMOMP/Z3-14, that shed over the course of the experiment were 69% (11 of 16) and 80% (12 of 15), respectively (p < 0.05; Table III). The nMOMP/A8-35 group was statistically different from its corresponding OVA-immunized controls (100%; p < 0.05) and not statistically different from C. muridarum-EB (29%; 4 of 14)–immunized control animals. As shown in Fig. 4A, the percentage of mice that shed in the nMOMP/A8-35–vaccinated group was significantly (p < 0.05) lower than in the OVA/A8-35–immunized group at day 4 (69

FIGURE 2. T cell–proliferative responses in the presence of nMOMP/A8-35 or nMOMP/Z3-14. To evaluate the possible toxic effects of nMOMP/A8-35 and nMOMP/Z3-14, T cells from naive mice were stimulated in vitro with Con A in the presence of the two nMOMP preparations. T cell–proliferative responses were determined by the increase in counts per minute (cpm). The values are means ± 1 SD of four different experiments. *p < 0.05 by Mann–Whitney rank-sum test compared with Con A, or Con A+nMOMP/A8-35, stimulated groups and p > 0.05 compared with medium-stimulated group. b p < 0.05 by Mann–Whitney rank-sum test compared with medium-stimulated group and p > 0.05 compared with Con A-stimulated group.

FIGURE 3. NMR studies: tryptophan side chains are more exposed when nMOMP is in complex with A8-35 compared with detergent. (A) Model of nMOMP (73) showing positions of seven of the eight Trp side chains at the interface of the lipid bilayer. One Trp occurs in VD4, which is not shown in this structural model. The 800-MHz NMR 15N HSQC spectra of 0.3 mM nMOMP solubilized in DPC (B) or trapped with A8-35 (C) in 90% H2O/10% D2O, 100 mM sodium phosphate (pH 7.4). Only Trp indole signals resonate in the [1H] region from 10 to 10.6. NMR data of 15N-labeled MOMP in the detergent DPC shows only one very weak Trp signal (B). In contrast, at least five strong Trp signals are seen when the protein is in APol A8-35 (C).
versus 100%), day 7 (62 versus 100%), day 10 (50 versus 100%), day 14 (31 versus 85%), and day 21 (0 versus 42%). At day 4 postchallenge, significantly fewer animals had positive vaginal cultures in the group vaccinated with nMOMP/Z3-14 when compared with the OV A/Z3-14–immunized mice (66 versus 100%; \( p < 0.05 \)). Furthermore, significantly fewer mice shed in the nMOMP/A8-35 vaccinated than in the nMOMP/Z3-14–immunized animals at day 14, 31 versus 73% (\( p < 0.05 \)).

A significant decrease (57%) in the length of time of shedding was observed in the nMOMP/A8-35–vaccinated animals (median days 12; range 4–21) when compared with the nMOMP/Z3-14–immunized mice (median days 21, range 4–35) or the OV A/A8-35–immunized mice (median days 28; 14–35) (\( p < 0.05 \)) (Table III). The negative control groups OV A/Z3-14 (median days 25, range 14–35), OV A/A8-35 (median days 28, range 14–35), or MEM-0 (median days 32, range 21–35), shed longer than the nMOMP–vaccinated animals, whereas the positive control \( C.\) muridarum–EB–immunized group had the shortest shedding time (median days 4, range 2–14).

There was also a statistically significant difference between the total number of positive vaginal cultures during the 6 wk of the experiment when comparing the nMOMP/A8-35– versus the nMOMP/Z3-14–vaccinated animals (Figs. 4A, 5). A total of 26.6% (34 of 128) of the vaginal cultures collected from the nMOMP/A8-35–vaccinated animals were positive, whereas 39.2% (47 of 120) of the cultures from the mice immunized with nMOMP/Z3-14 were positive (\( p < 0.05 \)). These values were statistically different from their respective negative control groups: OV A/A8-35 (57.7%; 60 of 104) and OV A/Z3-14 (56.3%; 54 of 96) or the MEM-0 (60.9%; 39 of 64). The positive \( C.\) muridarum–EB–immunized control group (6.3%; 7 of 112) was statistically significantly lower than any other group (\( p < 0.05 \)).

A significant reduction in the total number of \( C.\) muridarum IFUs per mouse recovered over the course of the experiment was observed in the groups vaccinated with nMOMP/Z3-14 (median 147,031; range 2–1,921,070) or nMOMP/A8-35 (median 14,125; range 2–2,817,998) when compared with their respective negative controls immunized with OV A/Z3-14, (median 2,719,510; range 272,555–9,862,992), or OV A/A8-35 (median 1,886,340; range 1,159,330–4,800,672), respectively (\( p < 0.05 \)) (Table III). No statistically significant difference was obtained when comparing the two nMOMP-vaccinated groups. The control \( C.\) muridarum–EB–immunized animals had the lowest total number of IFU/mouse (median 2; range 2–38,315) and that was statistically significant different from any other group (\( p < 0.05 \)).

The nMOMP/A8-35–vaccinated group shed significantly less IFU compared with the OV A/A8-35–immunized mice from day 4 (median number of IFU = 5,647 versus 486,324) through day 21 (median number of IFU = 2 versus 1,146) (\( p < 0.05 \)) (Fig. 5). The quantity of \( C.\) muridarum IFU shed by the group vaccinated with nMOMP/Z3-14 differed significantly from the OV A/Z3-14–immunized mice at day 4 (median number of IFU = 3,835 versus 1,600,234), day 7 (median number of IFU = 17,390 versus 1,110,582), and day 10 (median number of IFU = 46,168 versus 324,819) (\( p < 0.05 \)). Furthermore, the nMOMP/A8-35–vaccinated animals shed less than the nMOMP/Z3-14–immunized group on D10 (median number of IFU = 217 versus 46,168) and D14 (median number of IFU = 5.647 versus 1,146) (\( p < 0.05 \)). The median number of IFU shed by nMOMP/A8-35–vaccinated mice was below the limit of detection starting on D14 and all mice in this group had negative

**FIGURE 4.** Percentage of mice with positive vaginal cultures. (A) Percent immunized BALB/c mice with positive \( C.\) muridarum vaginal cultures. (B) Percent nMOMP/A8-35–vaccinated BALB/c mice, depleted and nondepleted of CD4+ or CD8+ T cells, with positive \( C.\) muridarum vaginal cultures. Following immunization mice were challenged i.vag. and vaginal cultures were collected over a 6-wk period. \( p < 0.05 \) by the Fisher’s exact test compared with the OVA/A8-35–immunized group; \( p < 0.05 \) by the Fisher’s exact test compared with the \( C.\) muridarum–EB–immunized group; \( p < 0.05 \) by the Fisher’s exact test compared with the OVA/Z3-14–immunized group; \( p < 0.05 \) by the Fisher’s exact test compared with the MEM-0–immunized group; \( p < 0.05 \) by the Fisher’s exact test compared with the nMOMP/Z3-14–immunized group; \( p < 0.05 \) by the Fisher’s exact test compared with the nMOMP/A8-35 no treatment group.
cultures by day 21, 2 wk earlier than the nMOMP/Z3-14–immunized animals.

**T cell depletion experiments**

To determine what type of T cell effects the protection elicited by the nMOMP/A8-35 vaccine, immunized mice were depleted before and after the vaginal challenge of CD4+ or CD8+ T cells using mAbs (Figs. 4B, 6). Before challenge, mice treated with the anti-CD8+ mAb had an 84.70% (1.86 of 12.5) ± 2.61 SD decrease in CD8 cells, whereas animals treated with the anti-CD4+ mAb had an 86.29% (3.5 of 25.54) ± 4.02 decrease in CD4+ cells when compared with the PBS control–inoculated group. Significantly more mice shed in the anti-CD4+ treated group when compared with nontreated group at day 21 (62 versus 0%) and onward (day 28: 50%, day 35: 37%, day 42: 25 versus 0%) (*p*, 0.05). In the CD4+ T cell–depleted group, there was an increase in length of days of shedding (median days 31.5; range 4–49) versus the nontreated (median days 12, range 4–21) and the CD8+ T cell–depleted (median days 9, range 4–28) groups (*p*, 0.05). Also, during the 6 wk of the experiment, there is a statistically significant difference (*p*, 0.05) between the total number of positive vaginal cultures in the CD4+ T cell–depleted group (60.9%; 39 of

**FIGURE 5.** Number of *C. muridarum* IFU recovered from the vagina following the i.vag. challenge. The number of *C. muridarum* IFU was quantitated for each individual culture. Dots represent individual animals, and the horizontal bars correspond to the medians. *p* < 0.05 by the Mann–Whitney rank-sum test compared with the OVA/Z3-14–immunized group. *p* < 0.05 by the Mann–Whitney rank-sum test compared with the *C. muridarum*–EB–immunized group. *p* < 0.05 by the Mann–Whitney rank-sum test compared with the MEM-0–immunized group. *p* < 0.05 by the Mann–Whitney rank-sum test compared with the OVA/A8-35–immunized group. *p* < 0.05 by the Mann–Whitney rank-sum test compared with the nMOMP/Z3-14–immunized group.
Table III. Results of vaginal cultures and fertility studies

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>No. of Mice Shed/ Total No. of Mice (%)</th>
<th>Total No. of IFU Shed/Mouse</th>
<th>Length of Shedding</th>
<th>No. of Fertile Mice/ No. of Embryos per Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median (Range)</td>
<td>Mean Days (Range)</td>
<td>Mean Total No. of Mice (%)</td>
</tr>
<tr>
<td>nMOMP/Z3-14</td>
<td>12/15 (80)</td>
<td>147,031^{\text{d}d}(&lt;2–1,921,070)</td>
<td>21^{\text{d}d}(4–35)</td>
<td>12/15^{\text{a}d}(80)</td>
</tr>
<tr>
<td>nMOMP/A8-35</td>
<td>11/16^{\text{b}b}(69)</td>
<td>14,125^{\text{c}c}(&lt;2–2,817,998)</td>
<td>12^{\text{c}c}(4–21)</td>
<td>12/16^{\text{c}c}(75)</td>
</tr>
<tr>
<td>OVA/Z3-14</td>
<td>12/10 (100)</td>
<td>2,719,510 (272,555–9,862,992)</td>
<td>25 (14–35)</td>
<td>7/12^{\text{c}c}(58)</td>
</tr>
<tr>
<td>OVA/A8-35</td>
<td>13/15 (100)</td>
<td>1,886,340 (1,159,330–4,800,672)</td>
<td>28 (14–35)</td>
<td>4/13^{\text{d}d}(31)</td>
</tr>
<tr>
<td>MEM-0</td>
<td>8/8 (100)</td>
<td>9,059,562 (894,734–13,003,396)</td>
<td>32 (21–35)</td>
<td>4/7 (57)</td>
</tr>
<tr>
<td>C. muridarum-EB</td>
<td>4/14 (29)</td>
<td>&lt;2(&lt;2–38,315)</td>
<td>4 (4–14)</td>
<td>14/14 (100)</td>
</tr>
</tbody>
</table>

Fertility control — — 14/16 (88) 4.44 4.44

*<p < 0.05 by the Fisher’s exact test compared with the C. muridarum-EB–immunized group.

*<p < 0.05 by the Fisher’s exact test compared with the OV A/A8-35–immunized group.

*<p < 0.05 by Mann–Whitney rank-sum test compared with OVA/Z3-14–immunized group.

*<p < 0.05 by Mann–Whitney rank-sum test compared with nMOMP/Z3-14–immunized group.

*<p < 0.05 by the Fisher’s exact test compared with the nMOMP/Z3-14–immunized group.

*<p < 0.05 by Mann–Whitney rank-sum test compared with the fertility control group.

*<p < 0.05 by the Fisher’s exact test compared with the fertility control group.

*<p < 0.05 by the Mann–Whitney rank-sum test compared with the nMOMP/Z3-14–immunized group.

*<p < 0.05 by the Mann–Whitney rank-sum test compared with the fertility control group.

*<p < 0.05 by the Mann–Whitney rank-sum test compared with the fertility control group.

64), versus the nontreated (26.6%; 34 of 128) and the CD8⁺ T cell–depleted (23.4%; 15 of 64) groups. In the CD8⁺ T cell–depleted and the nontreated groups, the total number of positive cultures was similar (p > 0.05). In addition, the nMOMP/A8-35–vaccinated group depleted of CD4⁺ T cells shed significantly higher numbers of IFU compared with either the nondepleted nMOMP/A8-35–vaccinated group at day 10 (median = 63,813 versus 2,16, day 14 (median = 4,832 versus <2), day 21 (median = 333 versus <2), day 28 (median = 3,365 versus <2), and day 35 (median = <2 versus <2) or with the anti-CD8 treatment group at day 14 (median = 4,832 versus <2) and day 21 (median = 333 versus <2) (p < 0.05).

Fertility studies
Six weeks after the intravaginal infection, mice were mated to determine the ability of the vaccine to protect against Chlamydia-induced infertility (Table III). The nMOMP/A8-35 (12 of 16; 75%) and the nMOMP/Z3-14 (12 of 15; 80%)–vaccinated groups had similar fertility rates when compared with the C. muridarum-EB (14 of 14; 100%) or the fertility control groups (14 of 16; 88%) or among themselves (p > 0.05). The nMOMP/A8-35–immunized group differed statistically from the OVA/A8-35 control (4 of 13; 31%; p < 0.05). The animals immunized with OVA/A8-35 (4 of 13; 31%) and OVA/Z3-14 (7 of 12; 58%) had statistically significant lower fertility rates when compared with the C. muridarum-EB group (p < 0.05).

The mean total number of embryos in nMOMP/A8-35 (3.75 ± 2.86)– or nMOMP/Z3-14 (4.87 ± 3.56)–immunized groups was no different from C. muridarum-EB (6.07 ± 2.16) or the fertility control groups (4.44 ± 2.76) or among themselves (p > 0.05; Table III). The nMOMP/A8-35–immunized group had statistically significant more embryos than the OVA/A8-35 control (1.31 ± 2.18). Mice immunized with OVA/A8-35 or OVA/Z3-14 (2.00 ± 1.91) had statistically lower number of embryos when compared with C. muridarum-EB immunized or the fertility control groups (p < 0.05).

Discussion
In this study, we tested the ability of a native MOMP preparation, formulated with a detergent (Z3-14) or an APol (A8-35), to induce in mice a protective immune response against an i.vag. challenge with C. muridarum. To elicit a Th1 response, Cpg-1826 and Montanide ISA 720 VG were used as adjuvants, and the vaccine was delivered by a combination of mucosal and systemic routes. Montanide was only used systemically. On the basis of the IgG2a/IgG1 Ab ratios in serum and IFN-γ levels in supernatants from stimulated splenocytes, both formulations induced robust Th1–biased responses. Overall, using native and heat-denatured EB as the Ag, the immune responses elicited by nMOMP/A8-35 were stronger than those achieved with nMOMP/Z3-14. Furthermore, using synthetic peptides corresponding to the amino acid sequence of MOMP, it was determined that more linear B cell epitopes were recognized by Abs elicited by vaccination with nMOMP/A8-35 than with nMOMP/Z3-14. Mice were challenged i.vag. with C. muridarum, and on the basis of the number of mice with positive vaginal cultures, length of shedding, total number of positive vaginal cultures and number of IFU recovered, the protection obtained with nMOMP/A8-35 was more robust than that achieved with nMOMP/Z3-14. Treatment of nMOMP/A8-35–vaccinated mice with an anti-CD4 Ab, but not with an anti-CD8 Ab, abrogated the protective effect of the vaccine. Fertility rates in nMOMP/A8-35 and nMOMP/Z3-14–immunized mice were comparable to those observed in the fertility control groups. The enhanced protection elicited by nMOMP/A8-35 may be the result of better accessibility of protective epitopes to the immune system in the APol nMOMP preparation versus the detergent solubilized protein.

Most of our current vaccines are based on live or inactivated whole pathogens and therefore maintain the native structure of their antigenic components (54, 55). As a result, upon exposure to a pathogen, the immune system of the vaccinated individual reacts with a well-directed response to the native Ags. New subunit vaccines, formulated with highly purified Ags that may lack the correct conformation, present new challenges (56–58). In order for a subunit vaccine to elicit a robust immune response, the structure of the Ag and accessibility of protective domains must be optimized (59, 60).

Using vaccine formulations with nMOMP/Z3-14, protection has been elicited in mice against genital and respiratory C. muridarum challenges and in nonhuman primates against a C. trachomatis ocular infection (23, 26, 28). However, as shown in this study, the detergent present in the nMOMP/Z3-14 preparation has a profound in vitro toxic effect that also could be, at high concentrations, detrimental in vivo (32, 33). In addition, detergents bound
to proteins are in a constant and rapid equilibrium with the solution (31). As a result, the Ag may change in conformation over time during storage and delivery of the vaccine and also when trafficking inside the body. For example, when membrane proteins are diluted under the critical micelle concentration of the detergent, as it would happen following injection into an individual, the protein aggregates and denaturation may occur (31, 34, 35). Both processes can result in epitopes becoming inaccessible or unrecognizable. Membrane protein–APol complexes, in contrast, are highly stable and do not dissociate following dilution, thus preventing protein aggregation (35, 61). Therefore, the nMOMP/ APol formulation should make for a safer delivery of the Ag to target cells while better maintaining the protein structure than the nMOMP/Z3-14 preparation (35, 62, 63). Furthermore, detergents and APols form around the transmembrane region of membrane proteins a belt into which hydrophobic protein surfaces are buried (64–68). Whether the belt is composed of detergent, or APols, has been shown to modulate the structure and dynamics of extramembrane protein loops, which can be expected to affect their antigenic properties (69). Finally, membrane proteins may be delivered in significantly different manners to cells of the immune system depending on whether they are surfactant free,
and possibly aggregated, as can be the case for detergent-based preparations, or kept soluble by the APol until delivered to a cell plasma or endocytic membrane (34, 35).

Membrane protein structures most often feature a ring of aromatic residues at the head-group region of the lipid bilayer (70). In fact, it has been shown thermodynamically that aromatic rings partition preferentially to the phosphocholine head-group region more favorably than any other amino acid group. Triptophan residues, in particular, have the largest ΔG of association with the phosphocholine head groups (71, 72). Similar to other membrane proteins, known porin structures display the aromatic ring distributions that favor head-group interactions. Although no structure of MOMP currently exists, four topological models of MOMP, corresponding to C. muridarum and the C. trachomatis serovars C, D, and F, have been proposed (73–76). All of these models position the seven or eight Trp rings in proximity to the lipid head-group region. One of these models is shown in Fig. 3 (73). On the basis of NMR measurements, we find that five Trp signals are solvent accessible in the APols preparation, but only one very weak Trp is detectable in DPC.

Because Z3-14 contains zwitterionic groups similar to phosphocholine, it is reasonable to suggest that the Z3-14 head group might favorably interact with Trp aromatic rings. In addition, Lys and Arg residues within MOMP extracellular loops could interact via salt bridges with the Z3-14 sulfate anion or the DPC phosphate anion. Both of these stabilizing interactions would create a situation where either Z3-14 or DPC detergent could efficiently occlude protein groups. Because the APol used in this study does not contain a zwitterionic group, or any feature that mimics lipid head-group distribution, it is not surprising that we find increased exposure for Trp residues normally buried at the head-group interface. MOMP displayed on the surface of Chlamydia would be expected to have even more protein buried because outer membranes are substantially thicker than the diameter of Z3-14 micelles. Thus, the accessible protein surface increases with the following order: Chlamydia-EB < nMOMP/Z3-14 < nMOMP/A8-35. Farris et al. (27) have shown that the protection elicited by nMOMP/Z3-14 is dependent on CD4+ and Abs but not on CD8+ T cells. In this study, we also showed that the protection elicited by the nMOMP/A8-85 is dependent on CD4+ but not on CD8+ T cells. The conformation of the Ag, and therefore accessibility to the immune system and processing, not only affects the epitopes that are recognized by Abs but also influences T cell responses. For example, Musson et al. (77) showed that the degree of Ag processing was dependent on the localization of the epitopes of the V. pestis Csp1 protein. Epitopes located in the globular domains were presented by newly synthesized MHC class II, after low pH-dependent lysosomal processing, whereas epitopes from a flexible strand of the protein were presented by mature MHC class II, independent of low pH, and did not require proteolytic processing. Warren et al. (78) also have described an antigenic peptide recognized by CD8+ T lymphocytes consisting of two noncontinuous peptide segments spliced in reverse order to that in the native protein. This type of modification could be affected by the conformation of the protein during proteasome processing. Furthermore, Tikhonova et al. (79) have shown that T cells, not undergoing MHC-specific thymic selection, can express TCRs that recognize conformational epitopes independently of MHC molecules.

In humans and mice, Th cell epitopes have been localized mainly to the constant domains (CD) of MOMP (46, 80). In this study, to determine which domains of MOMP function as Th cell Ags, we characterized MOMP peptides that elicited an IgG response following immunization with EB or the two MOMP preparations. Peptides corresponding to the four VD of MOMP were recognized by IgG Abs present in the serum from mice immunized with C. muridarum-EB and with nMOMP/A8-35 or nMOMP/Z3-14. Although Abs from mice immunized with EB only recognized the VD, sera from animals vaccinated with the nMOMP preparations also bound to peptides corresponding to the CD. Specific MOMP peptides in CD3, CD4, and CD5 were exclusively recognized by Abs from animals vaccinated with nMOMP/A8-35, and a different peptide in CD5 bound only to sera from mice immunized with nMOMP/Z3-14. The finding that a broader set of peptides was recognized by sera from mice immunized with nMOMP/A8-35 versus nMOMP/Z3-14 suggests that the accessibility of certain domains of MOMP to the immune system is different between the two formulations, a premise supported by our NMR data.

The increased accessibility of MOMP epitopes in the APols formulation to the humoral and cell-mediated immune systems may therefore explain our findings. Overall, higher neutralizing Ab levels, T cell–proliferative responses and levels of IFN-γ, were observed in mice immunized with nMOMP/A8-35 versus nMOMP/Z3-14. These may account for the more robust protection observed in the nMOMP/A8-35–vaccinated group of animals. The best protection, however, was achieved in mice immunized with live EB. The greater protection observed in these animals may be due to the presence in the EB of additional protective Ags, other than MOMP, and/or to better presentation of conformational epitopes of MOMP. In addition, EB replication following i.n. inoculation with live organisms results in dissemination of C. muridarum to most organs, including long-term colonization of the gastrointestinal tract, which could account for the more robust protection elicited in the control mice versus those vaccinated with nMOMP (81). However, implementation of a live Chlamydia vaccine is highly unlikely because of the potential induction of a hypersensitivity reaction upon exposure to this pathogen in EB-vaccinated individuals (2, 82). Furthermore, the complexities of growing and purifying large numbers of EB, safety concerns, and the high cost of production are significant limitations of a whole-organism vaccine. Therefore, there is a need to optimize our current subunit vaccine candidates.

In conclusion, we showed that nMOMP formulated with APols induces a very robust protection although, like almost all of our current vaccines, does not elicit sterilizing immunity (55). Using a computer model, Chlamydia vaccines with these characteristics have been shown to potentially have a major impact on the prevalence of these infections (83). Some new and under development subunit vaccines use integral membrane proteins that require components, such as detergents, to keep them in solution (59, 60). APols may be explored as an alternative to detergents to increase the safety and immunogenicity of these antigens.

Acknowledgments
We thank Prof. Rommie E. Amaro for the permission to use her three-dimensional model of MOMP (Fig. 3A).

Disclosures
The authors have no financial conflicts of interest.

References


