Liposome Delivery of Chlamydia muridarum Major Outer Membrane Protein Primes a Th1 Response That Protects against Genital Chlamydial Infection in a Mouse Model

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Background. Immunity to chlamydia is thought to rely on interferon (IFN)–γ-secreting T helper cells type 1 (Th1) with an additional effect of secreted antibodies. A need for Th1-polarizing adjuvants in experimental chlamydia vaccines has been demonstrated, and antigen conformation has also been reported as being important for raising protective immunity.

Methods. C57BL/6 mice vaccinated with native refolded Chlamydia muridarum major outer membrane protein (MOMP) adjuvanted with either Th1-promoting cationic adjuvant formulation 1 (CAF01) or T helper cells type 2–promoting aluminum hydroxide (alum) received a genital inoculation of $1.5 \times 10^5$ inclusion-forming units of C. muridarum. The role played by CD4+ T cells in MOMP/CAF01-raised immunity was investigated by depleting CD4+ T cells in vaccinated mice, and antigen conformation dependence was evaluated by vaccination with recombinant MOMP.

Results. Mice vaccinated with MOMP/alum displayed a strong anti-MOMP humoral response with high IgG1 titers, low levels of IFN-γ and tumor necrosis factor (TNF)–α, and only a slight reduction in chlamydial load. Mice vaccinated with MOMP/CAF01 displayed high titers of IgG2b, IFN-γ, and TNF-α and a profoundly reduced vaginal chlamydial load, compared with control mice. The protection was CD4+ T cell dependent and was not dependent on MOMP conformation.

Conclusion. CAF01 adjuvant facilitates a protective anti-MOMP CD4+ T cell response independent of MOMP conformation.

Chlamydia trachomatis infections are the most frequent sexually transmitted bacterial infections and are the causative agent of serious complications in infected women [1, 2]. Mouse models of chlamydial genital infections using the species Chlamydia muridarum have identified cell-mediated immunity (CMI) as being central to protection against disease and demonstrated a clear correlation between CD4+ T cells secreting interferon (IFN)–γ and protective immunity [3–6]. The protective roles of B cells and antibodies against chlamydia are not fully understood, and there are contrasting lines of evidence from studies of immune responses in infected individuals [7, 8]. In mice, antibodies were found not to play a role in the protective immune response during a primary infection [9] but clearly had a protective effect against a secondary challenge [10].

The major outer membrane protein (MOMP) is the target of both humoral and cellular immune responses during infections in humans [11, 12] and is a leading vaccine candidate. Despite the relative success of refolded native MOMP vaccines [13, 14], experimental vaccines based on recombinant MOMP (rMOMP), MOMP peptides, or MOMP DNA have not yielded the expected protection [15–18], and delivery systems that promote strong CMI as well as a conformation closer to the native preparation may be necessary for a protective immune response [19, 20]. Importantly, the choice of adjuvant for the refolded MOMP vaccine has a dramatic influence on vaccine efficacy. A recent study demon-
strated efficient protection with a vaccine that included Montanide ISA 720 and CpG-1826 as adjuvants [13], but another study of the same MOMP preparation combined with the adjuvants MF59, LT-K63, or LT-R72 failed to demonstrate any significant reduction in bacterial load [21]. In the present study, we compared the efficacy of C. muridarum MOMP adjuvanted with alum or the recently developed cationic liposome formulation 1 (CAF01; formerly termed DDA/TDB), which is currently in clinical development [22]. A product of Statens Serum Institut, Denmark, CAF01 is currently being evaluated in preclinical safety studies and promotes both strong cellular and humoral responses with a Th1 profile [23, 24]. Our study has demonstrated that, while alum-adjuvanted vaccine failed to reduce vaginal chlamydial load, refolded native MOMP as well as rMOMP combined with CAF01 induced a strong chlamydia-specific Th1 response, significantly reducing both the number of mice infected and the vaginal chlamydial load.

**METHODS**

**Organisms.** The C. muridarum strain MoPu/NiggII was purchased from the American Type Culture Collection and propagated in HLea 229 cells, as described elsewhere [25]. Chlamydia elementary bodies (EBs) were harvested, purified, and quantified as described elsewhere [26, 27] and were stored at −80°C in sucrose-phosphate-glutamate (SPG) buffer.

**MOMP preparation.** C. muridarum MOMP was extracted from infectious EBs by means of Zwittergent 3–14 (Calbiochem) and was purified on a hydroxylapatite (Calbiochem) column as described elsewhere [26, 28]. MOMP-containing fractions were identified by Western blot analysis using a polyclonal rabbit anti-MOMP antibody made in our laboratory. These fractions were pooled (figure 1A), and MOMP was refolded by dialysis against a glutathione buffer and fixed with glutaraldehyde, as described elsewhere [27, 28]. The refolded and fixed MOMP preparation was concentrated using a centrifugal filter unit (Centriprep YM-30; Millipore) and was dialyzed against PBS before injection. MOMP refolding was verified by SDS-PAGE as a change in migration from the denatured form of 42 kDa to a folded conformation of ~50 kDa (figure 1B).

**rMOMP.** The gene ompA (TC0052) was cloned and expressed in Escherichia coli, essentially as described elsewhere [29], and rMOMP was initially purified by metal chelate affinity chromatography, also essentially as described elsewhere [30, 31]. Further purification was done by ion exchange chromatography on a HiTrap Q Sepharose HP column (Amersham Biosciences). Purity was monitored by Western blot analysis, using antibodies against the His tag and E. coli (Dako). Fractions containing pure rMOMP were pooled and dialyzed against 20 mmol/L NH₄HCO₃ buffer (pH 8) with 10% glycerol and were stored at −20°C until use. The Limulus amebocyte lysate test (Endosafe; Charles River Laboratories) was used to determine the amount of endotoxin in the MOMP preparation, which was shown to be below the limit of detection (0.125 endotoxin units/mL).

**Western blot analysis.** Fifty micrograms of total protein from lysed C. muridarum EBs was electrophorized in a NuPAGE 4%–12% SDS-PAGE gel (Invitrogen). The proteins were blotted onto a nitrocellulose membrane, blocked, and, after thorough washing, incubated for at least 2 h with serum samples diluted 1:200. After extensive washing, alkaline phosphatase–conjugated rabbit anti–mouse immunoglobulin antibody (Dako), diluted 1:500 for 1 h, was used as secondary antibody, and membranes were developed using BCIP/NBT (Sigma).

**Mouse immunizations.** Female C57BL/6J mice, 4–5 weeks old, were purchased from Harlan Scandinavia. Animals were immunized subcutaneously with C. muridarum MOMP (5 µg/dose) in 100 µL of sterile PBS (pH 7.4), mixed by vortexing with either 100 µL of 2% Alhydrogel (Superfos; 500 µg/dose) or 100 µL of CAF01 adjuvant (Statens Serum Institut), which consisted of 50 µg of glycollaldehyde trehalose 6,6’-dibehenate per dose incorporated in 250 µg of cationic liposomes (composed of dimethyldioctadecylammonium) per dose. These amounts of antigen and adjuvant were titrated and found to be optimal in other experiments. Mice received a total of 3 immunizations at 2–week intervals. One week before C. muridarum challenge, the estrous cycle was synchronized by injection of 2.5 mg of medroxyprogesterone acetate (Depo-Provera; Pfizer). Six weeks after the final vaccination, the mice were challenged intravaginally with 1.5 × 10⁵ inclusion-forming units (ifu) (determined as 100 × ID₅₀ in our laboratory) of C. muridarum in 10 µL of SPG buffer. For intranasal infection, mice received the same infectious dose intranasally 8 weeks before vaginal challenge.

**Depletion of CD4⁺ T cells.** Monoclonal anti–mouse CD4 IgG2b (clone GK1.5) was purified from hybridoma supernatants made in our laboratory, using HiTrap Protein G HP columns (Amersham Biosciences). The purified IgG was dialyzed against PBS and sterile filtered, and the protein concentration was determined. Mice were depleted of CD4⁺ T cells via 5 injections of 0.5 mg of purified antibody each on days −8, −6, −4, −1, and 9 relative to the day of infection. The depletion of CD4⁺ T cells was verified by fluorescence-activated cell sorter (FACS) analysis on peripheral blood mononuclear cells (PBMCs) on days −1, 7, 15, and 42 relative to the day of infection. The depletion of CD4⁺ T cells had no impact on the size of the CD4⁺ T cell population (data not shown).

**Humoral anti-chlamydia response.** Blood was collected 7 days before infection and plasma was prepared for quantification of MOMP-specific antibodies by ELISA, essentially as described elsewhere [14]. Maxisorb plates (Nunc) were coated with rMOMP (0.5 µg/mL), and plasma samples were serially
diluted before being added to ELISA plates. MOMP-specific IgG1 and IgG2b were detected with isotype-specific horseradish peroxidase–conjugated rabbit anti–mouse IgG (Zymed), diluted 1:2000. The substrate was TMB PLUS (Kem-En-Tec). Reciprocal plasma dilutions corresponding to 50% maximal binding (i.e., EC50) were computed using Prism software (version 4; GraphPad).

**Chlamydia-specific cellular responses.** Three to 6 mice per group were killed 21 days after the final vaccination, and single-cell suspensions were prepared from individual spleens. Lymphocyte cultures were prepared essentially as described elsewhere [32] and were restimulated with heat-inactivated chlamydial EBs or rMOMP (5 μg/mL). Concanavalin A (5 μg/mL) and medium without antigen were used for positive and negative controls, respectively. After 72 h of incubation at 37°C in 5% CO2, supernatants were harvested and stored at −20°C. Interleukin (IL)–2, IL-4, IL-5, IFN-γ, and tumor necrosis factor (TNF)–α levels were then assayed using a mouse Th1/Th2 cytokine cytometric bead assay detection kit (BD Biosciences), in accordance with the manufacturer’s recommendations. Enzyme-linked immunospot (ELISpot) analysis was done in parallel, using 5 × 10^5 cells/well in 96-well MAHAS4510 plates (Millipore) that had been coated overnight at 4°C with 2 μg/mL rat anti–mouse IFN-γ antibody (BD Biosciences). Spots were

Figure 1. A, Silver-stained SDS-PAGE gel. Lane 1, molecular weight standard; lane 2, pooled major outer membrane protein (MOMP)–containing fractions after purification of MOMP from chlamydial elementary bodies (EBs). B, Coomassie-stained SDS-PAGE gel. Lane 1, molecular weight standard; lane 2, refolded MOMP prepared in nonreducing buffer; lane 3, refolded MOMP boiled in reducing buffer (lane 3). Refolding changes the migration of native MOMP to ~50 kDa, rather than the 42 kDa of denatured MOMP. C, Immunoblot of *Chlamydia muridarum* EB lysate probed with serum samples obtained 1 week before infection. Lane 1, molecular weight standard; lane 2, pool of serum samples from mice receiving saline, Th1-promoting cationic adjuvant formulation 1 (CAF01), or alum; lane 3, pool of serum samples from mice receiving MOMP/CAF01; lane 4, pool of serum samples from mice receiving MOMP/alum. D, EC50 of recombinant MOMP (rMOMP)–specific IgG1 and IgG2b in serum samples obtained 1 week before infection of mice vaccinated with MOMP/CAF01 (n = 17) or MOMP/alum (n = 9), or pooled data for mice that received saline (n = 7), CAF01 (n = 6), or alum (n = 7) (control pool, n = 20), as measured by ELISA. Results are representative of 3 independent experiments. E, Cytokine release, as measured in supernatants of splenocytes obtained 3 weeks after the final vaccination and restimulated with heat-inactivated *C. muridarum* EBs for 72 h. Interferon (IFN)–γ and tumor necrosis factor (TNF)–α levels are shown for mice vaccinated with MOMP/CAF01 (n = 6) or MOMP/alum (n = 6), as are pooled data from control mice treated with saline (n = 3), CAF01 (n = 3), or alum (n = 3) (control pool, n = 9). Results are representative of 3 independent experiments. **P < .01 (analysis of variance [ANOVA] with Dunnett’s posttest) for the comparison with the corresponding cytokine in the control pool. F, IFN-γ enzyme-linked immunospot (ELISpot) analysis of the same samples shown in panel E. Results are representative of 3 independent experiments. Spleens were obtained 3 weeks after final vaccination and were restimulated with heat-inactivated *C. muridarum* EBs for 48 h. Spots were visualized and counted using an automated ELISpot reader. Error bars in panels D–F indicate SEs. **P < .01 (ANOVA with Dunnett’s posttest) for the comparison between the no. of spots for the control pool and for MOMP/CAF01-vaccinated mice.

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developed and counted after 48 h of incubation using an automated ELISPOT reader, as described elsewhere [32].

**Vaginal chlamydial load.** Vaginal swab samples were obtained at 3, 7, 10, 14, and 21 days after infection. Samples were vortexed with glass beads in 1 mL of SPG buffer and stored at −80°C until analysis. Infectious loads were assessed by infection of McCoy cell monolayers with a titrated volume of the swap suspension. The plates were centrifuged at 750 g for 1 h at room temperature followed by incubation at 35°C for 2 h. Infection medium was then replaced with fresh medium, and the cells were incubated at 37°C for 40 h. Inclusions were visualized by staining with polyclonal rabbit anti-MOMP serum made in our laboratory, followed by FITC-conjugated swine anti-rabbit immunoglobulin (Dako). Background staining was done with propidium iodide (Invitrogen). Swap titrations were done in duplicate. Inclusions were enumerated by fluorescence microscopy, with observation of at least 10 individual fields of vision for each well.

**Gross pathologic changes.** Oviducts were subjected to macroscopic inspection for the presence of hydrosalpinx 6 weeks after infection.

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism software (version 4). Cytokine data were analyzed using analysis of variance (ANOVA) with Dunnett’s posttest. Median vaginal chlamydial loads were analyzed using the Kruskal-Wallis test and Dunn’s posttest, and the time to bacterial clearance was analyzed using the Kaplan-Meier test. The pathologic outcome of infection was analyzed using Fisher’s exact test.

**RESULTS**

**Immune response after vaccination.** The purity of the native MOMP preparation was visualized by means of silver-stained SDS-PAGE gels (figure 1A), and MOMP refolding was confirmed by a change in migration (figure 1B). Immunity after vaccination with MOMP/CAF01 and MOMP/alum was analyzed in 3 independent experiments, and representative results from 1 experiment are shown. The humoral response to vaccination was analyzed by Western blot analysis and ELISA. Western blot analysis of total chlamydial protein probed with pools of serum samples from mice vaccinated with MOMP/CAF01 (figure 1C, lane 3) or MOMP/alum (figure 1C, lane 4) revealed a single band at 42 kDa corresponding to MOMP, thus confirming the purity of the native MOMP preparation, whereas a pool of serum samples from mice receiving saline, CAF01, or alum (figure 1C, lane 2) did not recognize any chlamydial proteins. Anti-rMOMP IgG2b levels were highest in the MOMP/CAF01-vaccinated mice (average EC50, 2.1 log; n = 17), and IgG1 levels were highest in the MOMP/alum-vaccinated mice (average EC50, 2.7 log; n = 9) (figure 1D). No rMOMP-specific IgG1 or IgG2b was detected in serum samples from individual control mice (n = 20) treated with saline (n = 7), CAF01 (n = 6), or alum (n = 7) (figure 1D).

Three to six mice per group were killed 3 weeks after final vaccination. Cytokines released by splenocytes on stimulation with heat-inactivated chlamydial EBs were analyzed by cytometric bead assay (IL-2, IL-4, IL-5, IFN-γ, and TNF-α) and ELISPOT (IFN-γ) assay. Splenocytes from MOMP/CAF01-vaccinated mice (n = 6) released high levels of IFN-γ (mean ± SE, 4,851 ± 1784 pg/mL) and TNF-α (mean ± SE, 1,403 ± 300 pg/mL) on restimulation with inactivated EBs (figure 1E) or rMOMP (data not shown). These levels were significantly higher than those measured in mice that received saline, CAF01, or alum (control pool, n = 9) (P < .01, ANOVA), whereas no significant differences in IFN-γ or TNF-α levels were found in samples from mice vaccinated with MOMP/alum. IL-2, IL-4, and IL-5 levels did not change significantly in any of the vaccinated groups, compared with the control pool (data not shown). ELISPOT analysis showed a significant higher number of IFN-γ-producing splenocytes in the MOMP/CAF01 group (mean ± SE, 483 ± 129 spots/1 × 10⁶ cells) than in a pool of mice treated with saline, CAF01, or alum (control pool) (P < .01), whereas the MOMP/alum-vaccinated group did not differ significantly from control mice (mean ± SE, 16 ± 5 spots/1 × 10⁶ cells) (figure 1F). In a separate experiment, intranasally infected mice were completely protected against a secondary vaginal infection with C. muridarum (data not shown), in accordance with findings of other studies [14, 33]. The anti-rMOMP antibody profile and titers in these mice were found to be similar to those in an MOMP/CAF01-vaccinated group, with high anti-rMOMP IgG2b titers (average EC50, 3.2 log) and low anti-rMOMP IgG1 titers (average EC50, 0.9 log). In vitro stimulation of splenocytes with heat-inactivated chlamydial EBs induced IFN-γ release at a level similar to that seen in a group vaccinated with MOMP/CAF01.

**Protection against vaginal challenge with chlamydia conferred by MOMP/CAF01 vaccination.** We began by comparing the protective effects of MOMP/CAF01 and MOMP/alum vaccinations. Mice were vaccinated and, 6 weeks later, challenged intravaginally with 1.5 × 10⁵ ifu of C. muridarum. Vaginal swab samples were obtained on days 7, 14, and 21. MOMP/CAF01 had a clear protective effect; compared with mice treated with saline, mice immunized with MOMP/CAF01 were completely protected against a secondary vaginal infection with C. muridarum (data not shown), in accordance with findings of other studies [14, 33]. The anti-rMOMP antibody profile and titers in these mice were found to be similar to those in an MOMP/CAF01-vaccinated group, with high anti-rMOMP IgG2b titers (average EC50, 3.2 log) and low anti-rMOMP IgG1 titers (average EC50, 0.9 log). In vitro stimulation of splenocytes with heat-inactivated chlamydial EBs induced IFN-γ release at a level similar to that seen in a group vaccinated with MOMP/CAF01.
confirm that the protection observed in the MOMP/CAF01-vaccinated group was not due to adjuvant-induced nonspecific responses, the alum (figure 3A) and CAF01 (figure 3B) adjuvants were compared with saline in a separate experiment and found not to differ significantly (table 2).

**Dependency of vaccine-mediated protection against chlamydia on CD4+ T cells.** We continued by investigating the CD4+ T cell contribution to the protective immunity raised by MOMP/CAF01 vaccination. Mice vaccinated with MOMP/CAF01 were depleted of CD4+ T cells via injection of anti-mouse CD4 monoclonal antibodies on days −8, −6, −4, −1, and 9 relative to infection. Injection of irrelevant rat IgG2b (isotype control) in a separate group of mice had no impact on the number of CD4+ T cells (data not shown). FACS analysis of blood showed that the CD3+CD4+ cell population of total PBMCs in mice receiving GK1.5 was 0.2% one day before infection (17% in nondepleted mice), and 6 weeks after infection this number had increased to 6.9% (13.7% in nondepleted mice). Mice receiving the MOMP/CAF01 vaccine (n = 7) had significantly reduced chlamydial loads on days 3 (P < .01) and 7 (P < .05) (figure 4A and table 3), whereas mice receiving an identical vaccine followed by CD4+ T cell–depleting antibodies (n = 6) did not have reduced chlamydial loads at any time point (figure 4B and table 3). On days 7 (P < .05) and 10 (P < .01), there were significant differences in chlamydial load between vaccinated mice and vaccinated and depleted mice (table 3) The percentages of culture-positive MOMP/CAF01-vaccinated mice were 100%, 57%, 43%, and 29% on days 3, 7, 10, and 14, compared with 100%, 100%, 100%, and 83%, respectively, for the vaccinated and depleted mice, but infection-resolution times did not differ significantly between these groups.

**Similar levels of protection provided by vaccination with refolded native MOMP/CAF01 and rMOMP/CAF01.** Protective immune responses to MOMP have been described elsewhere as being dependent on conformation [14] and not induced efficiently by

### Table 1. Protective effect of major outer membrane protein (MOMP)/Th1-promoting cationic adjuvant formulation 1 (CAF01) and MOMP/alum against *Chlamydia muridarum* infection (data from experiment 1).

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<thead>
<tr>
<th>Treatment group</th>
<th>Days after infection</th>
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<td></td>
<td>7</td>
</tr>
<tr>
<td>Saline</td>
<td></td>
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<tr>
<td><em>C. muridarum</em> shed</td>
<td>6.375 (6.151–6.733)</td>
</tr>
<tr>
<td>Proportion (%) positive</td>
<td>10/10 (100)</td>
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<tr>
<td>MOMP/alum</td>
<td></td>
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<tr>
<td><em>C. muridarum</em> shed</td>
<td>6.195 (5.708–6.703)</td>
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<tr>
<td>Proportion (%) positive</td>
<td>10/10 (100)</td>
</tr>
<tr>
<td>MOMP/CAF01b</td>
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</tr>
<tr>
<td><em>C. muridarum</em> shed</td>
<td>4.544 (1.0–5.785)</td>
</tr>
<tr>
<td>Proportion (%) positive</td>
<td>7/9 (78)</td>
</tr>
</tbody>
</table>

**NOTE.** Data for *C. muridarum* shedding are median (range) log_{10} *C. muridarum* ifu shed per mouse, and data for positivity are the no. of culture-positive mice per the total no. of mice (%). Data are representative of 3 independent experiments. Culture-negative mice were assigned the lower cutoff of the shedding assay (10 ifu/mouse).

- P < .05 for the comparison of vaginal chlamydial load between saline-treated and vaccinated mice (Kruskal-Wallis test, Dunn’s posttest).
- P = .0092 for the comparison of the no. of mice shedding chlamydia after genital infection (infection-resolution time) between MOMP/CAF01- and saline-treated groups (Kaplan-Meier test).
- P < .01 for the comparison of vaginal chlamydial load between saline-treated and vaccinated mice (Kruskal-Wallis test, Dunn’s posttest).
- P < .001 for the comparison of vaginal chlamydial load between saline-treated and vaccinated mice (Kruskal-Wallis test, Dunn’s posttest).

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**Figure 2.** Vaccine-induced protection (data from experiment 1). Mice were vaccinated 3 times at 2-week intervals and then vaginally infected with *Chlamydia muridarum* (1.5 × 10^5 ifu/mouse) 6 weeks after the final vaccination. Median vaginal bacterial loads are compared for mice receiving saline (n = 10) vs. mice receiving major outer membrane protein (MOMP)/alum (n = 10) (A) or MOMP/Th1-promoting cationic adjuvant formulation 1 (CAF01) (n = 9) (B) at 7, 10, 14, and 21 days after infection. Culture-negative mice were assigned the lower cutoff of the shedding assay (10 ifu/mouse). Table 1 provides more data from experiment 1.
recombinantly expressed protein [15, 16, 34–36]. However, our demonstration that the MOMP/CAF01 vaccine effect was mediated primarily by CD4+/H11001 T cells encouraged us to investigate whether a protective response could also be achieved via immunization with C. muridarum rMOMP/CAF01. IFN-γ and TNF-α titers on re-stimulation of PBMCs or splenocytes from these mice were similar to those observed in mice that received the refolded native MOMP/CAF01 vaccine (data not shown). Mice vaccinated with refolded native MOMP (figure 5A) or rMOMP (figure 5B) adjuvanted in CAF01 were almost equally protected against vaginal infection compared with mice treated with CAF01 alone. Median chlamydial loads were significantly reduced in MOMP/CAF01-vaccinated mice, compared with those in mice treated with CAF01 alone, on days 3, 7, 10, and 14 (P < .05) after infection (table 4), and mice immunized with rMOMP/CAF01-vaccinated mice, the infection-resolution time was significantly reduced, compared with the CAF01 control group (P = .0078) (table 4).

**Gross pathologic changes.** As a measure of infection-induced pathology, the number of mice in which unilateral or bilateral hydrosalpinx had developed was evaluated 6 weeks after challenge. Considering all 3 experiments together, 47% of mice receiving saline, 55% of MOMP/CAF01-vaccinated mice, and 42% of MOMP/alum-vaccinated mice had hydrosalpinges 6 weeks after infection. The pathologic outcome in the vaccine groups were therefore not significantly different from that in the saline group (Fisher’s exact test).

**DISCUSSION**

Chlamydial MOMP contains both B and T cell epitopes and is probably the chlamydia vaccine antigen that has been tested most extensively in different adjuvant systems and efficacy experiments. Although MOMP is a promising antigen, it has been difficult to develop it into a vaccine product; major obstacles include the need for clinical-grade adjuvants to promote CMI and the potential need for MOMP preparations with the natural conformation. Conformational MOMP epitopes have been suggested to be important by experiments demonstrating that a refolded native MOMP vaccine partially lost its protective effect after MOMP denaturation [14], and this observation was also

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**Table 2.** Protective effect of alum and Th1-promoting cationic adjuvant formulation 1 (CAF01) alone against *Chlamydia muridarum* infection (data from experiment 3).

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<th>Treatment group</th>
<th>Days after infection</th>
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<tr>
<td><strong>Saline</strong></td>
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<tr>
<td>Proportion (%) positive</td>
<td>7/7 (100)</td>
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<tr>
<td><strong>Alum</strong></td>
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<tr>
<td><em>C. muridarum</em></td>
<td>6.280 (5.378–6.452)</td>
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<tr>
<td>Proportion (%) positive</td>
<td>7/7 (100)</td>
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<tr>
<td><strong>CAF01</strong></td>
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<tr>
<td><em>C. muridarum</em></td>
<td>6.312 (5.792–6.670)</td>
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<td>Proportion (%) positive</td>
<td>6/6 (100)</td>
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**NOTE.** Data for *C. muridarum* shedding are median (range) log_{10} *C. muridarum* ifu shed per mouse, and data for positivity are the no. of culture-positive mice per the total no. of mice (%). No significant differences were detected between the control treatment groups in vaginal *C. muridarum* load or infection-resolution time. Culture-negative mice were assigned the lower cutoff of the shedding assay (10 ifu/mouse).
supported by the finding that vaccines based on rMOMP have in general failed to induce significant levels of protection [15, 16, 34–36]. The importance of MOMP conformation for protective immunity is intriguing, because protection against chlamydial infection is generally believed to rely mainly on specific CD4+ T cells and IFN-γ secretion, which in turn are considered to be dependent on linear epitopes. Therefore, the failure of rMOMP vaccines may relate to the overall CMI/humoral profile of the response induced in these vaccination experiments. Mouse experiments have shown that refolded native MOMP administered in CMI-promoting adjuvants, such as Freund’s complete adjuvant [14] or Montanide ISA 720 plus CpG-1826 adjuvant [13], or expressed in Vibrio cholerae ghosts [37] promotes an efficient protective immune response against vaginal challenge with chlamydia, whereas MOMP combined with adjuvants known to induce a humoral response, such as alum (figure 2A) or MF59 [21], produced only very limited protective effects.

In the present study, we have demonstrated that the CAF01 adjuvant combined with C. muridarum MOMP primes a CD4+ T cell—dependent protective response that is independent of MOMP conformation. CAF01 is a recently developed adjuvant that consists of liposomes and a synthetic mycobacterial cord factor as an immune modulator [23]. CAF01 promotes both strong CMI and antibody response and was demonstrated to have major potential in experimental malaria and tuberculosis vaccines [24, 38]. This adjuvant is currently on track to enter human clinical trials in the near future. It has been demonstrated recently that, in murine Mycobacterium tuberculosis vaccine experiments, the CAF01 adjuvant primes a strong vaccine-specific memory response consisting of polyfunctional (IFN-γ+, TNF-α+, and IL-2+) CD4+ T cells that mediate protection against M. tuberculosis as late as 14 months after vaccination (T. Lindgren, E.M.A., K. Korsholm, I. Rosenkrands, and P.A., unpublished data). In the present study, the MOMP-specific immunity

Table 3. Protective effect of major outer membrane protein (MOMP)/Th1-promoting cationic adjuvant formulation 1 (CAF01), with and without CD4+ T cell depletion, against Chlamydia muridarum infection (data from experiment 2).

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<tr>
<td>CAF01</td>
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<td>C. muridarum shed</td>
<td>8.380 (7.048–8.665)</td>
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<tr>
<td>Proportion (%) positive</td>
<td>7/7 (100)</td>
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<tr>
<td>MOMP/CAF01, no depletion</td>
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<tr>
<td>C. muridarum shed</td>
<td>6.230 (4.595–7.736)</td>
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<tr>
<td>Proportion (%) positive</td>
<td>7/7 (100)</td>
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<tr>
<td>MOMP/CAF01, CD4+ T cell</td>
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<tr>
<td>depletion</td>
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<td>C. muridarum shed</td>
<td>6.685 (5.966–7.956)</td>
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<td>Proportion (%) positive</td>
<td>6/6 (100)</td>
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</table>

NOTE. Data for C. muridarum shedding are median (range) log10 C. muridarum ifu shed per mouse, and data for positivity are the no. of culture-positive mice per the total no. of mice (%). No significant difference in infection-resolution time between groups was detected (Kaplan-Meier). Data are representative of 2 independent experiments. Culture-negative mice were assigned the lower cutoff of the shedding assay (10 ifu/mouse).

a P < .01 for the comparison of vaginal chlamydial load between CAF01 and vaccine (Kruskal-Wallis test, Dunn’s posttest).

b P < .05 for the comparison of vaginal chlamydial load between CAF01 and vaccine (Kruskal-Wallis test, Dunn’s posttest).

c P = .05 for the comparison of vaginal chlamydial load between CAF01 and vaccine (Kruskal-Wallis test, Dunn’s posttest).

d P < .01 for the comparison of vaginal chlamydial load between CAF01 and vaccine (Kruskal-Wallis test, Dunn’s posttest).
induced by the MOMP/CAF01 vaccine was also characterized by a strong cellular response, with high levels of IFN-γ and TNF-α (figure 1E and 1F) as well as high titers of systemic IgG2b (figure 1D), indicative of a Th1 immune response [24]. This response was able to protect against vaginal challenge with chlamydia (figures 2, 4, and 5) and was not due to adjuvant-induced nonspecific responses (figure 3 and table 2). Levels of IgG2b and IFN-γ after MOMP/CAF01 vaccination reached the same magnitude found in animals after nasal challenge with C. muridarum, a protocol that has been reported elsewhere [14, 33] and confirmed in our laboratory to promote complete protection against C. muridarum intravaginal challenge.

CMI protection against challenge with chlamydia was clearly demonstrated by depleting the CD4+ T cell subset after vaccination and before challenge with chlamydia, which resulted in an almost complete loss of protection (figure 4B). The alum-based vaccine used for comparison mainly induced a Th2 response, with high anti-chlamydia IgG1 titers and low IFN-γ and TNF-α levels (figure 1D–1F), and failed to protect against vaginal challenge (figure 2A). We also showed that, in contrast to the disappointing results obtained elsewhere with rMOMP in adjuvants that drive a predominantly humoral response, rMOMP in CAF01 promotes the same level of protection as refolded native MOMP (figure 5B).

Our data, therefore, strongly point to the importance of CMI responses to nonconformational epitopes as the major effector mechanism of the protective immune response seen in MOMP/CAF01-vaccinated animals. However, although bacterial replication was effectively controlled in MOMP/CAF01-vaccinated mice, we still found significant pathology in this group 6 weeks after infection, whereas animals primed by a prior intranasal challenge were also completely protected against the development of pathology. Several studies have shown that specific antibodies are an important component in the first phase of an efficient secondary immune response against chlamydia [5, 10], and our data from the depletion experiment suggest some early (3 days after infection) CD4+ T cell–independent immunity that may involve antibodies (figure 4B). High levels of MOMP-

Table 4. Protective effect of major outer membrane protein (MOMP)/Th1-promoting cationic adjuvant formulation 1 (CAF01) and recombinant MOMP/CAF01 against Chlamydia muridarum infection (data from experiment 3).

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Days after infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>C. muridarum shed</td>
<td>6.312 (5.792–6.670)</td>
</tr>
<tr>
<td>Proportion (%) positive</td>
<td>6/6 (100)</td>
</tr>
<tr>
<td>MOMP/CAF01</td>
<td>4.856 (3.731–6.187)</td>
</tr>
<tr>
<td>Proportion (%) positive</td>
<td>8/8 (100)</td>
</tr>
<tr>
<td>rMOMP/CAF01</td>
<td>5.241 (4.244–6.475)</td>
</tr>
<tr>
<td>Proportion (%) positive</td>
<td>9/9 (100)</td>
</tr>
</tbody>
</table>

NOTE. Data for C. muridarum shedding are median (range) log10 C. muridarum ifu shed per mouse, and data for positivity are the no. of culture-positive mice per the total no. of mice (%). Culture-negative mice were assigned the lower cutoff of the shedding assay (10 ifu/mouse). rMOMP, recombinant MOMP.

a P = .05 for the comparison of vaginal chlamydial loads between CAF01 alone and vaccine (Kruskal-Wallis test, Dunn’s posttest).
b P = .0078 for the comparison of the no. of mice shedding chlamydia after genital infection (infection-resolution time) between rMOMP/CAF01 and CAF01 alone (Kaplan-Meier test).
specific antibodies on the mucosal surface in animals with a strong CMI response to MOMP may promote increased uptake of antibody-coated chlamydial EBs in Fc receptor–positive antigen-presenting cells, leading to accelerated triggering of T cell immunity, as suggested by recent findings in Fc receptor knockout mice [39]. Such an antibody-mediated triggering and accelerated involvement of the protective CMI response may ensure optimal early control of the invading pathogen and reduced pathology.

In conclusion, the present study shows that a vaccine based on a novel adjuvant inducing both strong CMI and antibody responses. Bio- nically refolded as well as recombinantly expressed MOMP administered in a CMI-promoting adjuvant such as CAF01 induces a CD4+ T cell–dependent immune response that efficiently protects against vaginal challenge with C. muridarum. Increasing the levels of IgG2a/b on mucosal surfaces to accelerate recall responses and reduce pathology is the aim of ongoing work.

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References


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