

CD4⁺ T Cells and Antibody Are Required for Optimal Major Outer Membrane Protein Vaccine-Induced Immunity to *Chlamydia muridarum* Genital Infection[∇]

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Despite effective antimicrobial chemotherapy, control of *Chlamydia trachomatis* urogenital infection will likely require a vaccine. We have assessed the protective effect of an outer membrane protein-based vaccine by using a murine model of chlamydial genital infection. Female mice were first vaccinated with *Chlamydia muridarum* major outer membrane protein (MOMP) plus the adjuvants CpG-1826 and Montanide ISA 720; then they were challenged with *C. muridarum*. Vaccinated mice shed 2 log₁₀ to 3 log₁₀ fewer inclusion-forming units (IFU) than ovalbumin-vaccinated or naïve animals, resolved infection sooner, and had a lower incidence of hydrosalpinx. To determine the relative contribution of T cells to vaccine-induced protection, mice were vaccinated, depleted of CD4⁺ or CD8⁺ T cells, and then challenged vaginally with *C. muridarum*. Depletion of CD4⁺ T cells, but not depletion of CD8⁺ T cells, diminished vaccine-induced protection, with CD4-depleted mice shedding 2 log₁₀ to 4 log₁₀ more IFU than CD8-depleted or nondepleted mice. The contribution of antibodies to vaccine-induced protection was demonstrated by the absence of protective immunity in vaccinated B-cell-deficient mice and by a 2 log₁₀ to 3 log₁₀ decrease in bacterial shedding by mice passively administered an anti-MOMP serum. Thus, optimal protective immunity in this model of vaccine-induced protection depends on contributions from both CD4⁺ T cells and antibody.

New cases of sexually transmitted diseases number more than 340 million worldwide annually and pose a formidable health risk to infected individuals (67–69). It is estimated that *Chlamydia trachomatis*, the causative agent of chlamydia, is responsible for more than 92 million of these cases. In the United States, where *C. trachomatis* infections are the infections most commonly reported to the Centers for Disease Control and Prevention, there are more than 4 million new cases each year (14, 67). As a bacterial agent of infection, *C. trachomatis* can be eradicated efficiently with appropriate antibiotic treatment, but more than 50% of infected individuals are asymptomatic and therefore lack the impetus to seek treatment (14). When left untreated, infection in women can lead to pelvic inflammatory disease, ectopic pregnancy, and tubal factor infertility and can cause severe and sometimes irreparable damage to the reproductive organs (14, 67). To combat the high rate of infection and disease, the development of an efficacious vaccine is critical.

Trachoma vaccine trials using whole organisms in the 1950s and 1960s had mixed results, with some studies inducing only partial, serovar-specific, short-lived immunity (4). In one study, a subset of vaccine trial participants experienced an increased incidence of disease and exacerbated pathology relative to that of their unvaccinated counterparts upon reexposure to chlamydiae, which led many researchers to abandon the use of

whole organisms in immunizations (7, 8). Since then, no other human vaccine trials targeting ocular or urogenital *C. trachomatis* infections have been published. Instead, researchers have focused their efforts on animal models of ocular and genital infection.

To this end, the murine model of chlamydial genital infection, which closely mimics acute genital infection in women, has been employed extensively for the study of the immunological parameters of infection and for vaccine development. Mice infected with *C. muridarum* naturally resolve infection in approximately 4 weeks and develop long-lived adaptive immunity that markedly protects against reinfection (3, 37). Infection elicits *Chlamydia*-specific CD4⁺ T cells, CD8⁺ T cells, and antibody, but only CD4⁺ T cells are necessary for resolution of the primary infection (42). In contrast, immunity to reinfection is dominated by both protective CD4⁺ T cells and antibody (38). Clearance and immunity are highly dependent on a Th1-type response, specifically gamma interferon (IFN-γ)-secreting CD4 cells (9). On the other hand, Th2 responses are associated with scarring and immunopathology (62). For example, antibody responses dominated by IgG1 are not protective and may be associated with an increase in pathology, whereas anti-chlamydial antibodies of the IgG2a and IgG2b isotypes are associated with a protective response (51).

Using knowledge of the protective response gleaned from the murine model of infection-induced immunity, investigators have made modest strides toward the development of an efficacious vaccine. Studies utilizing whole elementary body (EB) immunization have induced significant protection, though most of these studies have limited real-world application. One notable example that induced almost sterilizing immunity involved the passive transfer of dendritic cells pulsed ex

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vivo with nonviable chlamydiae (65). Subunit antigen vaccines represent the bulk of vaccine studies, and vaccines based on combinations of a number of chlamydial antigens, adjuvants, and delivery systems have had varying degrees of success in preventing infection (8, 22, 62). Chlamydial antigens, including secreted proteins, such as chlamydial protease-like activity factor (CPAF) (16, 33, 43–46), and membrane associated proteins, such as PorB (26, 30) and IncA (33), have also been used in subunit vaccines; however, the vast majority of studies have focused on the major outer membrane protein (MOMP), an immunodominant antigen in both human and animal studies (22, 62). Novel delivery systems, including *Vibrio cholerae* ghosts and cationic liposomes, have been introduced into chlamydial vaccine research, and while initial studies have shown incomplete protection, these systems may have the potential to elicit protective responses against chlamydial genital infection when used in conjunction with appropriate antigens (2, 19, 20, 23).

Despite substantial effort, no vaccine licensed for human use is currently available. Recently, a MOMP-based vaccine utilizing the adjuvants CpG-1826 and Montanide ISA 720, which together drive a strong Th1-type response, has been shown to confer considerable protection when mice are challenged directly in the upper genital tract with *C. muridarum* (51). In our current study, we sought to determine if this vaccine protected against vaginal challenge (the natural route of infection) and to evaluate the contributions of T cells and antibody to the vaccine-induced protective response. We found that the MOMP vaccine conferred significant protection against vaginal challenge and protected against infection-induced pathology (hydrosalpinx). Furthermore, optimal protection was dependent on both CD4⁺ T cells and antibody.

MATERIALS AND METHODS

Mice. Female C57BL/6 mice and B6.129S2-*Igh-6^{mi}Cg^{nl}*/J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were maintained in the animal facilities at the University of Alabama at Birmingham or the University of Arkansas for Medical Sciences (Little Rock, AR). B6.129S2-*Igh-6^{mi}Cg^{nl}*/J mice, referred to below as B-cell-deficient mice, have a disruption in the membrane exon of the immunoglobulin μ chain gene, rendering them unable to produce mature B cells; they are, therefore, antibody negative (32). Five- to 15-week-old mice were used throughout these studies. All animal procedures were approved by the institutional animal care and use committees.

Bacterial growth, purification, and enumeration. *Chlamydia muridarum* (strain Weiss) was grown in HeLa 229 cells and was purified by discontinuous density gradient centrifugation (10). The infectivity titer was determined on HeLa cell monolayers as previously described (10).

Preparation of *C. muridarum* MOMP. MOMP was purified as previously described (51). Briefly, HeLa 229 cells were infected with *C. muridarum* at a multiplicity of infection (MOI) of 1.25, and 39 h later, they were washed with Hanks balanced salt solution, sonicated, and then centrifuged at 30,000 \times g for 1 h. The pellet was resuspended in phosphate-buffered saline (PBS) and was then treated with 25 μ g/ml DNase I for 1 h at 4°C. Following centrifugation for 1 h at 75,600 \times g, the pellet was extracted twice in 0.2 M phosphate buffer (pH 5.5) containing 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 100 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM EDTA at 37°C and was centrifuged for 1 h at 75,600 \times g between extractions. The pellet was then extracted in the phosphate buffer described above, with 2% Anzergent 3-14 (Z3-14) (Anatrace, Maumee, OH) in place of CHAPS at 37°C, and was then centrifuged for 1 h at 75,600 \times g. The supernatant was applied to a 20-cm by 1.5-cm hydroxyapatite column (Bio-Gel HTP; Bio-Rad, Hercules, CA) that had been equilibrated with 0.02 M phosphate buffer (pH 5.5) with 0.1% Z3-14 and was eluted in a linear gradient from 0.02 M to 0.6 M the same phosphate buffer. Fractions exhibiting an increase in absorbance at 280 nm were analyzed by sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE), and those containing a major band at 40 kDa were pooled. The purified MOMP preparation reacted with an anti-MOMP monoclonal antibody (Mo33b) (17) and with convalescent serum by immunoblotting and exhibited a banding pattern, as shown previously (51).

Purified MOMP was refolded by dialysis in 0.1 M phosphate buffer with 0.05% Z3-14, 2 mM reduced glutathione, 1 mM oxidized glutathione, and 1 mM EDTA. The protein was fixed with 2% glutaraldehyde for 2 min at room temperature, and then the fixation was stopped with 2 M glycine. The protein was concentrated with a Centrplus filter (Millipore, Billerica, MA) and was dialyzed against 0.02 M phosphate buffer (pH 7.4) with 0.15 M NaCl and 0.05% Z3-14 prior to immunization.

Immunization and challenge of mice. Animals were immunized with *C. muridarum* MOMP by the subcutaneous (s.c.) (10 μ g/mouse) and intramuscular (i.m.) (10 μ g/mouse) routes, boosted twice with the same immunization at 2-week intervals, and then rested for 4 to 6 weeks. The vaccine consisted of MOMP plus CpG oligodeoxynucleotide 1826 (5'TCCATGACGTTCCGACGT T-3') (Invivogen, San Diego, CA) and Montanide ISA 720 (Seppic, Inc., Fairfield, NJ) at a 30:70 (vol/vol) ratio of MOMP plus CpG to Montanide, with a final CpG concentration of 10 μ g/mouse per injection site. The preparation was emulsified with a syringe. A control group was immunized as described above with 10 μ g/mouse ovalbumin in place of MOMP to mimic the control groups of other studies (29, 51). Mice that had resolved a prior genital infection with *C. muridarum* served as immune positive controls (infection-immune group). A group of naïve animals was challenged as a negative control. Five days prior to infectious challenge, mice were injected s.c. with 2.5 mg medroxyprogesterone acetate (SICOR Pharmaceuticals, Irvine, CA). On the day of challenge, the vaginal vault was gently swabbed with a calcium alginate-tipped swab (Fisher Scientific, Waltham, MA) to remove excess mucus. Mice were then inoculated vaginally with 5 μ l containing 5×10^4 inclusion-forming units (IFU) (equivalent to 100 50% infectious doses [ID₅₀]) of *C. muridarum*. The infection was monitored by enumerating the number of IFU recovered from cervicovaginal swabs using indirect immunofluorescence as previously described (37).

***In vivo* T-cell subpopulation depletion.** Mice were depleted of CD4⁺ or CD8⁺ T cells as previously described (40). Briefly, mice were injected intraperitoneally (i.p.) with 400 μ g of an anti-CD4 (clone GK 1.5) or anti-CD8 (clone 2.43) monoclonal antibody on the following schedule, where day 0 is the day of infectious challenge: days -6, -5, -4, -1, 2, 5, 8, 11, 14, 17, 20, and 23. This depletion regimen effectively depleted either CD4⁺ or CD8⁺ T cells for the entire 4-week test period (42).

Passive transfer of serum. Two sources of sera, designated convalescent and anti-MOMP sera, were used for passive immunization studies. Convalescent serum is defined as serum collected from mice that had resolved a primary chlamydial genital infection (infection immune). Anti-MOMP serum is defined as serum collected from mice that had been immunized with the MOMP vaccine as described above. Mice were injected i.p. with 0.5 ml of convalescent or anti-MOMP serum on the following schedule, where day 0 is the day of secondary infectious challenge: days -1, 0, 3, 6, 10, and 14. Normal, nonimmune serum had been tested previously in passive transfer experiments and was shown not to confer any level of protection (38). Therefore, those studies were not repeated here.

***Chlamydia* ELISA.** Antibody titers were determined by enzyme-linked immunosorbent assays (ELISA) using *C. muridarum* EBs as the antigen (37). Serum used in passive transfer experiments was evaluated prior to transfer. Mice were also bled 10 days after challenge in order to assess antibody levels in mice receiving passively administered serum. Vaginal wash specimens were collected 2 weeks after final vaccination or 43 days postinfection and were analyzed as previously described (37).

***Chlamydia*-specific cytokine responses of splenocytes.** Four weeks after the final vaccine booster or infection with *C. muridarum*, animals were sacrificed, and spleens were collected. Splenocytes from each mouse were mixed with UV-inactivated *C. muridarum* EBs at a ratio of 1:4 (cells to EBs), and the mixtures were incubated at 37°C for 1 h with tumbling. The splenocyte-EB mixture was then added to a 96-well flat-bottom tissue culture plate at 5×10^5 cells/well and was incubated for 72 h at 37°C. Supernatants were collected, clarified by centrifugation, and analyzed for interleukin-2 (IL-2), IL-4, IL-5, IL-10, IL-12(p70), granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor alpha (TNF- α), and IFN- γ by using a Bio-Plex Pro Th1/Th2 assay (Bio-Rad) according to the manufacturer's protocol.

Statistical analysis. Two-way analysis of variance (ANOVA) with a Bonferroni posttest was used to analyze the difference between IFU counts of control and experimental groups. Differences in hydrosalpinx incidence were analyzed by a chi-square test.

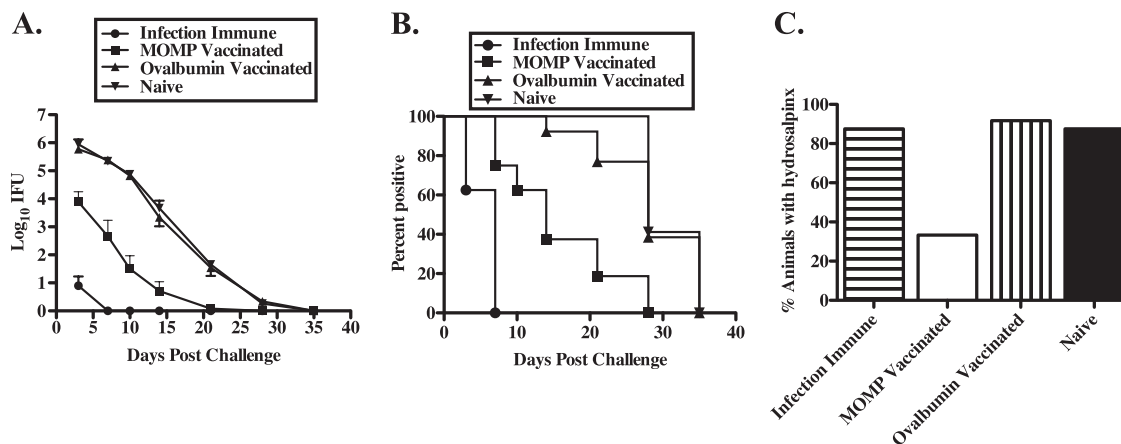


FIG. 1. The MOMP vaccine protects in a vaginal model of genital infection. Female naïve, infection-immune, MOMP-vaccinated, and ovalbumin-vaccinated C57BL/6 mice were challenged intravaginally with 100 ID₅₀ of *C. muridarum*. (A) Course of infection, represented as log₁₀ chlamydial IFU recovered from cervicovaginal swabs ± standard errors of the means. (B) Percentage of culture-positive mice per group at each culture time point. (C) Percentage of mice with unilateral or bilateral hydrosalpinx at 70 days postinfection. The data represent duplicate determinations for groups of at least 5 mice each.

RESULTS

MOMP vaccination provides significant protection against vaginal challenge and lowers the incidence of hydrosalpinx. To determine if MOMP vaccination protected against vaginal challenge, mice were vaccinated with MOMP plus CpG-1826 and Montanide ISA 720 and were then challenged intravaginally with *C. muridarum*. MOMP-vaccinated animals shed 2 log₁₀ to 3 log₁₀ fewer IFU throughout the course of infection than either ovalbumin-vaccinated negative-control or naïve mice (P , <0.001 at days 3, 7, 10, 14, and 21) (Fig. 1A). MOMP vaccination resulted in a shortened duration of infection, with 79% of mice clearing infection by 21 days postchallenge versus 24% of ovalbumin-vaccinated and none of the naïve mice (Fig. 1B). MOMP vaccination did not protect as well as prior infection, which induced immune responses that dramatically reduced the shedding of infectious bacteria and shortened the course of infection (P , <0.001 at days 3, 7, and 10; P , <0.05 at day 14) (Fig. 1A and B). Additionally, 40% of infection-immune animals were solidly protected against reinfection, whereas all MOMP-vaccinated animals were infected (Fig. 1B, day 3).

Hydrosalpinx, the accumulation of fluid in the oviduct due to the occlusion of the oviduct by scar tissue, is a measurement of infection-induced pathology that results from chlamydial genital tract infection. To determine if the MOMP vaccine protected against the hydrosalpinx that develops subsequent to lower genital tract infection, animals were sacrificed 70 days post-infectious challenge, and their genital tracts were assessed for the presence of hydrosalpinx. Significantly fewer MOMP-vaccinated animals than animals in the control groups developed either unilateral or bilateral hydrosalpinx (P , <0.0001) (Fig. 1C). Although the infection-immune group was protected against rechallenge, the high incidence of hydrosalpinx was expected, because the pathology is a result of the primary infection with *C. muridarum*, and therefore, the rate should be similar to that of the naïve group. Collectively, MOMP vaccination conferred significant protection against *C. muridarum* vaginal challenge, as demonstrated by reduced bacterial shed-

ding, a shortened duration of infection, and a reduced incidence of hydrosalpinx.

MOMP vaccine-induced immune responses. To assess the humoral immune response elicited following MOMP vaccination, sera from MOMP-vaccinated animals were analyzed by ELISA and immunoblotting, and the results were compared to those for convalescent serum. MOMP-vaccinated animals developed high-titer anti-chlamydial IgG1, IgG2b, IgG2c, and IgG3. The responses were similar to those of convalescent serum except for the IgG1 response, which was substantially higher in the anti-MOMP serum, and the IgA response, which was not detected in anti-MOMP serum (Fig. 2A). C57BL/6 mice have IgG2c rather than IgG2a (36), but both were included in the analysis, because the reagents used to detect IgG2a and IgG2c cross-react, and historically IgG2a has been measured rather than IgG2c. Immunoblotting confirmed that sera from vaccinated animals reacted with the MOMP used in the vaccine as well as with the MOMP in whole *C. muridarum* EBs, and convalescent serum recognized the MOMP used in the vaccine (51) (data not shown). Local anti-chlamydial Ig and IgA were detected in secretions from infection-immune mice, whereas MOMP-vaccinated mice had anti-chlamydial Ig but no IgA (Fig. 2B).

Cytokine responses of splenocytes from MOMP-vaccinated, infection-immune, ovalbumin-vaccinated, and naïve mice were measured using a multiplex Th1/Th2 cytokine assay. Splenocytes from infection-immune animals produced IL-4, GM-CSF, and IFN- γ when stimulated with chlamydiae (Fig. 3), while specific responses from vaccinated animals were limited to IFN- γ (Fig. 3C). No other antigen-specific cytokine responses were detected (data not shown).

CD4⁺ T cells, but not CD8⁺ T cells, contribute to the MOMP vaccine-induced protective response. In an effort to elucidate the roles of CD4⁺ and CD8⁺ T cells in the vaccine-induced protective response, we used monoclonal antibodies to deplete CD4⁺ and CD8⁺ cells in vaccinated animals. As seen in Fig. 4A, MOMP-vaccinated mice depleted of CD8⁺ T

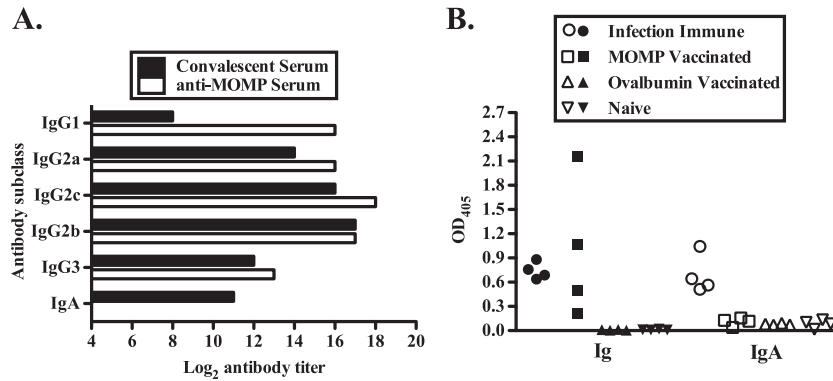


FIG. 2. Vaccination elicited a *Chlamydia*-specific antibody response. (A) Anti-chlamydial antibody titers of pooled anti-MOMP sera and pooled convalescent sera as determined by ELISA. (B) Anti-chlamydial antibody responses of vaginal wash fluids as determined by ELISA. Data are presented as optical densities (OD) for vaginal wash fluids from individual mice. Filled symbols represent the Ig response, and open symbols represent the IgA response.

cells prior to challenge had a course of infection similar to that of nondepleted MOMP-vaccinated animals. In contrast, depletion of CD4⁺ T cells resulted in significantly greater bacterial shedding and infections of longer duration than those of either nondepleted or CD8⁺ T-cell-depleted vaccinated mice ($P, <0.001$ at days 7, 10, 14, and 21; $P, <0.01$ at day 28) (Fig. 4A and B). The incidence of hydrosalpinx in CD4-depleted MOMP-vaccinated animals (63.6%) was more than twice that in nondepleted animals (30%), while no increase was observed in CD8-depleted animals (40%). Thus, CD4⁺ T cells, but not CD8⁺ T cells, contribute importantly to the vaccine-induced protective response.

Antibody and CD4⁺ T cells are required for optimal MOMP vaccine-induced immunity. Depletion of CD4⁺ T cells in vaccinated animals diminished protective immunity but did not completely ablate the animals' ability to control the infection (Fig. 4). Therefore, since CD8⁺ T cells do not apparently contribute to the protective response (Fig. 4), we sought to determine if antibody contributed significantly to vaccine-induced immunity. To test the effect of antibody deficiency on

vaccine-induced protection, B-cell-deficient mice were vaccinated with MOMP or ovalbumin and were then challenged vaginally with *C. muridarum*. Naïve B-cell-deficient mice, which lack mature B cells and are therefore antibody negative, resolve *C. muridarum* genital infection similarly to wild-type immunocompetent animals (Fig. 1A) and develop significant immunity to reinfection (Fig. 5A) (42). The immunity that develops in antibody-deficient mice following *C. muridarum* genital infection is dependent on CD4⁺ T cells, since mice depleted of these cells are unable to resolve the infection (Fig. 5A). The course of infection in MOMP-vaccinated B-cell-deficient animals was similar to those for both naïve and ovalbumin-vaccinated mice, indicating that antibody plays an important role in the vaccine-induced protective response ($P, <0.05$ at all days assessed) (Fig. 5B). MOMP-vaccinated antibody-deficient mice also were not protected against the development of hydrosalpinx; more than 80% of these animals developed bilateral or unilateral hydrosalpinx.

Having found that both CD4⁺ T cells and antibody contribute to the vaccine-induced protective response (Fig. 4 and 5),

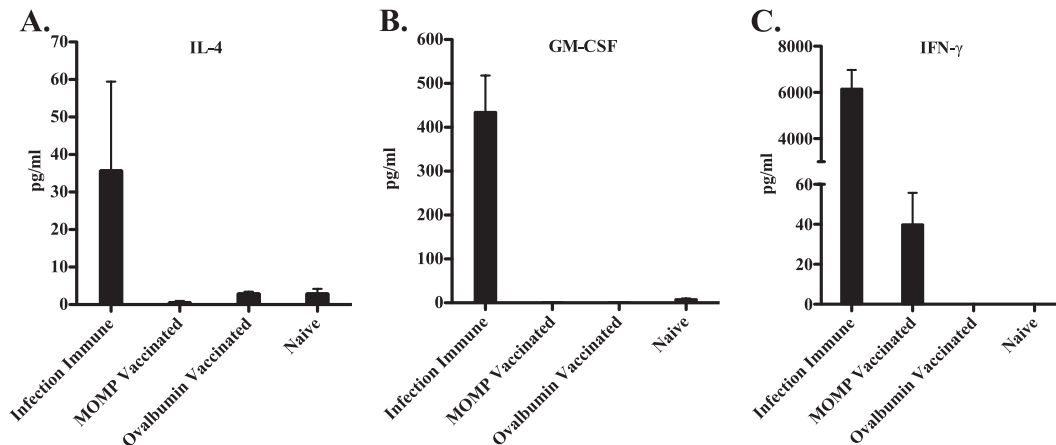


FIG. 3. Cytokine responses of splenocytes stimulated with UV-inactivated *C. muridarum* EBs. Splenocytes were collected from mice 4 weeks after infection or final vaccination and were then stimulated with *C. muridarum* EBs for 72 h. Supernatants were assessed for cytokines using a BioPlex Pro Th1/Th2 cytokine assay. Concentrations represent the cytokine concentration minus the background and are expressed as the average concentration for each group in pg/ml.

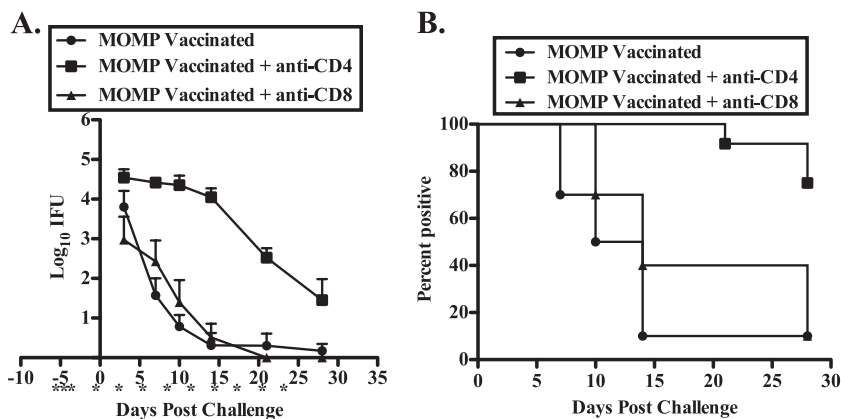


FIG. 4. CD4⁺, but not CD8⁺, T cells are involved in the MOMP vaccine-induced protective response. Female C57BL/6 MOMP-vaccinated mice were either left untreated or depleted of CD4⁺ or CD8⁺ T cells prior to intravaginal infection with 100 ID₅₀ of *C. muridarum*. Depletion was maintained throughout the course of infection as indicated (*). (A) Course of infection, expressed as log₁₀ IFU ± standard error of the mean. (B) Percentage of culture-positive mice at each time point. The data represent duplicate determinations for groups of least 5 mice.

we next wanted to assess the state of the protective response in the absence of both of these components. Thus, MOMP-vaccinated B-cell-deficient mice were depleted of CD4⁺ T cells and challenged. The initial course of infection in these mice was similar to that of infection-immune B-cell-deficient mice depleted of CD4⁺ T cells; mice in both groups shed high numbers of IFU for the first 2 weeks postinfection (Fig. 5A and B). However, bacterial shedding decreased in the MOMP-vaccinated group beginning 2 weeks postinfection and continued to decrease throughout the observation period (Fig. 5B). Bacterial shedding remained significantly higher in this group than in the naïve and nondepleted groups of vaccinated animals (Fig. 5B). Collectively, these data indicate that both CD4⁺ T cells and antibody are necessary for optimal MOMP vaccine-induced protection.

Anti-MOMP serum offers significant protection against reinfection. The B-cell-deficient mice not only are deficient in

antibody but also lack other, antibody-independent B-cell responses (e.g., antigen presentation) (32). Therefore, we do not know if the diminished protective response elicited by MOMP vaccination of B-cell-deficient mice was due to the lack of specific antibody or to another B-cell function. To specifically assess the contribution of antibody to the vaccine-induced protective response, we used an established infection model of B-cell-deficient mice, which has proven ideal for assessing the protective efficacy of passively transferred serum (38). Infection-immune B-cell-deficient mice were depleted of CD4⁺ T cells, passively administered convalescent or anti-MOMP serum, and then challenged intravaginally with *C. muridarum*. The titers and Ig class/subclass characteristics of convalescent and anti-MOMP sera were determined by ELISA both prior to passive transfer and 10 days postchallenge (Fig. 6A and B). Because these studies were performed using B-cell-deficient mice, the antibody detected 10 days post-infectious challenge

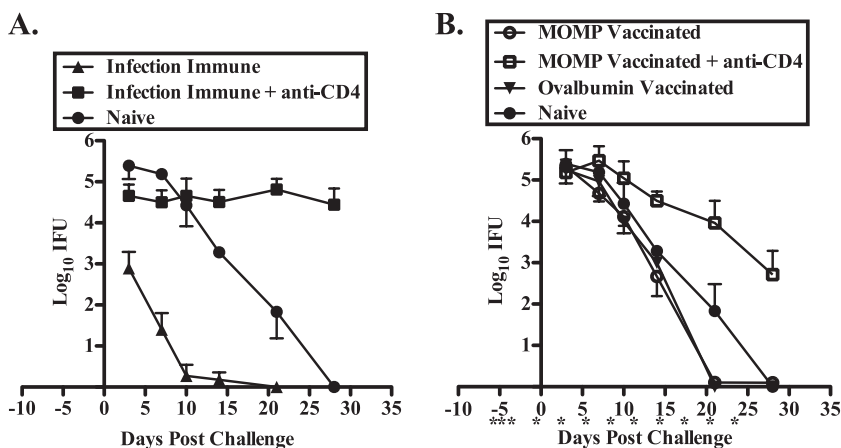


FIG. 5. CD4⁺ T cells and antibody contribute to the MOMP vaccine-induced protective response. Female B-cell-deficient mice were separated into naïve, infection-immune, MOMP-vaccinated, and ovalbumin-vaccinated groups and were then either left untreated or treated with anti-CD4 prior to challenge with 100 ID₅₀ of *C. muridarum*. Panels A and B represent results for animals from the same experiment, but the graphs are separated for clarity. Also, the course of infection in naïve animals is depicted on both graphs for comparison. (A) Courses of infection of naïve mice, infection-immune mice, and infection-immune mice treated with anti-CD4. (B) Courses of infection of naïve mice, MOMP-vaccinated mice, MOMP-vaccinated mice treated with anti-CD4, and ovalbumin-vaccinated mice. Data are presented as log₁₀ IFU ± standard errors of the means of duplicate determinations for at least 5 mice.

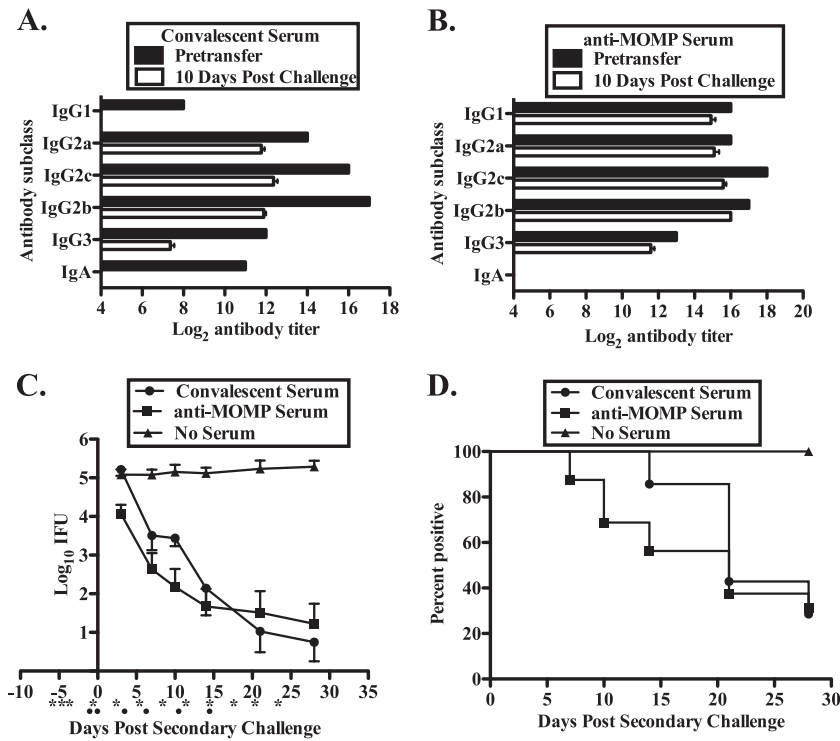


FIG. 6. Passive transfer of protective immunity with anti-MOMP serum. Convalescent and anti-MOMP sera were evaluated by ELISA prior to passive transfer and 10 days post-secondary infection. (A and B) Anti-chlamydial titers for convalescent serum (A) and anti-MOMP serum (B), presented as log₂ titers, prior to passive transfer and 10 days after reinfection. Pretransfer anti-chlamydial titers represent duplicate determinations for pooled convalescent or anti-MOMP sera, and day 10 postinfection data represent the mean titer for at least 10 mice per group. (C and D) B-cell-deficient mice that had resolved a primary *C. muridarum* genital infection were first treated with anti-CD4 and were then either injected i.p. with convalescent or anti-MOMP serum or left untreated, as described in Materials and Methods. All mice were infected intravaginally with 100 ID₅₀ of *C. muridarum*, and the course of infection was monitored. (C) Course of infection, expressed as log₁₀ IFU ± standard error of the mean of duplicate determinations for at least 5 mice. (D) Percentage of culture-positive animals at each time point tested.

reflects the passively transferred antibody and is not due to a response to infection. Notable differences between the convalescent and anti-MOMP sera include the lack of IgA in the anti-MOMP serum and the low IgG1 titer in the convalescent serum (Fig. 2 and 6). Following passive transfer, antibody titers diminished somewhat, and anti-chlamydial IgA was undetectable in the convalescent serum, as noted previously (38).

Infection-immune B-cell-deficient mice depleted of CD4⁺ T cells were unable to resolve infection and shed 10⁵ chlamydiae for the duration of the experiment (Fig. 6C). In contrast, mice receiving either convalescent or anti-MOMP serum shed far fewer chlamydiae (2 log₁₀ to 4 log₁₀ fewer) at most time points tested (*P*, <0.05 at days 7 and 10 and <0.001 at days 14, 21, and 28 for convalescent serum; *P*, <0.05 at day 7 and <0.001 at days 10, 14, 21, and 28 for anti-MOMP serum). Furthermore, 50% of the anti-MOMP serum-treated mice and 70% of the convalescent serum-treated mice completely resolved the infection (Fig. 6D). These data clearly demonstrate the important role of antibody in MOMP vaccine-induced immunity.

DISCUSSION

The protective efficacy of an anti-chlamydial vaccine is judged by its ability to prevent both infection and infection-related sequelae. Parenteral vaccination with MOMP provides partial protection against vaginal challenge, as evidenced by a

decrease in bacterial shedding and a lowered incidence of hydrosalpinx (Fig. 1) (51). Historically, two models of genital tract infection have been used to assess vaccines: upper genital tract challenge via the ovarian bursa and lower genital tract challenge via the vagina. In women, the natural route of infection with *C. trachomatis* initiates at the cervix, thus favoring the model of lower genital tract challenge for vaccine research. Vaginal challenge allows researchers to assess the ability of a vaccine to prevent infection and, in turn, the immunopathological damage to the genital tract resulting from infection. Although the upper genital tract infection model does not reproduce the natural infection route, it is quite useful for assessing the potential of a chlamydial vaccine to prevent infertility. Direct inoculation of chlamydiae into the upper genital tract increases the incidence of infertility from a primary infection. We and others have shown that parenteral vaccination with MOMP is protective in both upper and lower genital tract challenge models. MOMP vaccination shortens the course of infection, decreases bacterial shedding, and decreases the incidence of hydrosalpinx and/or infertility (Fig. 1) (51).

The importance of T cells in natural immunity to chlamydial genital infections has long been established (58, 59). Gene knockout mice, passive transfer of immune T cells, and *in vivo* depletion of T-cell subsets have demonstrated that CD4⁺ T

cells are the predominant protective T-cell subset (37, 42, 64). *In vivo* cytokine depletion has been used to demonstrate that cytokines produced by CD4⁺ T cells of the Th1 lineage, such as IFN- γ and IL-12, are important for protection, while those produced by Th2 lineage cells, such as IL-4, are not protective and may lead to an exacerbation of the infection and an increase in pathology (61). In our current study, we used the adjuvants CpG-1826 and Montanide ISA 720 along with native MOMP to drive a strong Th1-type response, and we found that protection was dependent on CD4⁺ T cells but not on CD8⁺ T cells (Fig. 4). Similarly, protection following vaccination with recombinant MOMP was found to be CD4⁺ T-cell dependent (23). Although not all chlamydial vaccines eliciting Th1-type responses are protective, those that decidedly elicit Th1-type responses confer the most significant protection (7, 22). Studies utilizing adjuvants that induce Th2-type responses have not been as successful (22, 50, 51, 53).

Research from the past 20 years has produced data that challenge the paradigm of immunological duality, which posits that immunity to intracellular pathogens is dependent on cell-mediated responses, while antibody-mediated responses confer immunity to extracellular pathogens (11, 12). Several studies clearly show that antibody does provide a level of protective immunity to intracellular pathogens (6, 13, 38). Monoclonal antibodies raised to surface-exposed chlamydial antigens, such as MOMP, and convalescent serum have been shown to neutralize the infectivity of *C. trachomatis* and *C. muridarum* in *in vitro* cell infection assays (34, 37, 54, 55, 57, 72). Investigators have also noted an initial decrease in infectivity *in vivo* when EBs are coated with anti-MOMP monoclonal antibodies prior to vaginal challenge in mice and ocular infection in monkeys, and this limited protection is thought to be a result of direct neutralization of the pathogen (57, 73). Similar results have been reported for *Mycobacterium bovis* when bacteria are coated with monoclonal antibodies before intranasal inoculation (1). In contrast to the protection seen in studies with *Mycobacterium tuberculosis* (1), passive transfer of anti-chlamydial monoclonal antibodies prior to and after infectious challenge does not provide significant protection against infection, and this is particularly obvious in the absence of CD4⁺ T cells (17, 38, 57). The lack of protection against chlamydial genital infection conferred by passive transfer of convalescent serum and monoclonal antibodies has been attributed to the magnitude and composition of the local response. Earlier studies suggested that passive transfer may not result in the localization of sufficient antibody to the genital tract (57). However, more-recent work has shown that the passive transfer of monoclonal antibodies or convalescent serum does confer protection against reinfection and that antibody from the passively administered serum can be found in the vaginal secretions (38). It should be noted, however, that antibody-mediated protection is entirely dependent on CD4⁺ T-cell-mediated adaptive changes in the genital tract tissues but is not dependent on direct interaction with CD4⁺ T cells (38). We do not yet fully understand what comprises the adaptive changes that are needed for antibody to confer protection against genital *Chlamydia* infection. However, our studies indicate that protection appears not to be mediated by antibody directly neutralizing chlamydiae or simply through the interaction of antibody with chlamydiae and acute inflammatory cells. The adaptive

changes that we believe are necessary for antibody to confer protection appear to be due either to recruitment and/or to activation of an effector cell population by CD4⁺ T cells. One possible mechanism would include the recruitment and activation of NK cells, which could confer protection through antibody-dependent cellular cytotoxicity. At this time, however, the precise mechanism by which antibody protects is unknown.

Using the model of genital tract reinfection in B-cell-deficient mice, we found that antibody contributed to the MOMP vaccine-induced protective response (Fig. 5). Vaccinated B-cell-deficient mice were not protected, and the course of infection in those mice was similar to those in naïve and ovalbumin-vaccinated animals. B cells have functions in addition to antibody production (e.g., antigen presentation); thus, it was important to determine whether the failure of the MOMP vaccine to protect B-cell-deficient mice was due to the absence of mature B cells or to the lack of antibody (21, 31). The passive transfer of anti-MOMP serum conferred a level of protective immunity on B-cell-deficient mice, indicating that antibody contributes to protective immunity in this vaccine model (Fig. 6). Collectively, our results provide further evidence that an efficacious vaccine against chlamydial genital infection should elicit both protective cell-mediated CD4⁺ T-cell and humoral immune responses.

MOMP vaccination resulted in significant protection against chlamydial vaginal challenge compared to nonvaccinated or ovalbumin-vaccinated negative controls, but it did not protect as well as infection-based immunity (Fig. 1A). This is particularly obvious in early infection (days 3 and 7), when infection-immune mice shed 3 log₁₀ fewer IFU than MOMP-vaccinated mice. This result is in contrast to the protection observed in a cynomolgus monkey model of ocular infection. Monkeys immunized with *C. trachomatis* MOMP prepared similarly to the MOMP used in the current study displayed immunity superior to that elicited following infection (29). The difference between the levels of protective immunity elicited by MOMP vaccination in the two models is not understood but could be due to the model (mouse versus monkey or genital versus ocular infection), the pathogen (*C. muridarum* versus *C. trachomatis*), or the immune responses. One key difference, however, is that in mice, *C. muridarum* causes robust infection with marked inflammation (39). This marked inflammation, and specifically that mediated by CD4⁺ T cells, is thought to be essential for optimal immunity (38). During genital infection, epithelial cells infected with chlamydiae respond by secreting proinflammatory and immunoregulatory cytokines, which are important in driving protective Th1 immunity (8, 60). IL-18, for instance, is secreted by the infected epithelial cells and, in turn, interacts with IL-12, promoting the differentiation of the Th1 subset of helper T cells and inducing the production of IFN- γ , which is important for the clearance of, and immunity to, genital tract infection (8, 18, 27, 28, 56).

Although optimal immunity is conferred by a prior *C. muridarum* genital infection, infection and vaccination of distant mucosal sites have been shown to protect against genital challenge. Immunizing mice intranasally with inactivated *C. muridarum* results in long-lasting protection against genital challenge (48, 49). Mucosal vaccination with MOMP encapsulated with vault nanoparticles, which appear to target dendritic cells, induced protective immunity against genital challenge, with

significant decreases in bacterial shedding at all time points tested, including points as early as day 3 postchallenge (15). In the current study, the MOMP vaccine was administered parenterally rather than mucosally, which likely explains the delayed protection observed in vaccinated mice compared to infection-immune mice (Fig. 1A). Although mucosal vaccination appears to elicit strong protective responses against genital challenge, at present no mucosal adjuvants have been approved for use in humans. Another notable example of a vaccine that induced remarkable protective immunity without resulting in inflammation in the genital tract was the passive transfer of dendritic cells pulsed *ex vivo* with inactivated *C. muridarum* (65). While this type of personalized medicine will not likely come to fruition as a prophylactic treatment for a nonlethal infection, it does demonstrate the usefulness of dendritic cells in vaccine strategies targeting *Chlamydia* and other pathogens (15, 24, 35, 70, 71).

The type and magnitude of the immune response are important factors that determine the quality of the protective response. We found that MOMP vaccination elicited cellular and humoral responses that differed from those elicited following infection. Splenocytes from MOMP-vaccinated mice produced significantly less IFN- γ than splenocytes from infection-immune animals (Fig. 3C). In addition to the differences in the magnitude of the response, the type of response elicited by vaccination also differed from that elicited by infection. A weak IFN- γ response was the only antigen-specific response detected from EB-stimulated splenocytes from MOMP-vaccinated animals, while infected animals produced IL-4 and GM-CSF in addition to IFN- γ (Fig. 3). Antibody responses also differed in that vaccinated animals produced substantially more IgG1 than infection-immune animals, and they did not produce *C. muridarum*-reactive IgA (Fig. 2). Although these differences could explain the disparity in protection observed following MOMP vaccination versus infection, they did not impact the ability of passively transferred antiserum to protect (Fig. 6). Furthermore, deficiency in IgA does not impact the course of *C. muridarum* infection in mice (41).

Other MOMP-based vaccines (5, 19, 20, 25, 63) that induce strong cellular and humoral Th1 type responses have not conferred the same level of protection as we, and others, have observed (7, 8, 29, 51), and the structural conformation of the MOMP antigen may explain the difference in the level of protection. Although the crystal structure of MOMP has yet to be resolved, Pal et al. have shown that a preparation of refolded native MOMP is more protective than recombinant MOMP (47, 50–53). A comparison of the refolded MOMP vaccine, as was used in the current study, to a preparation containing recombinant MOMP with the same adjuvants supports this notion (66). Likewise, dendritic cells pulsed *ex vivo* with recombinant MOMP are not protective, whereas dendritic cells pulsed with nonviable EBs are markedly protective (63).

In summary, we have shown that parenteral MOMP vaccination confers a significant level of protective immunity to genital challenge. Protection is dependent on both CD4⁺ T cells and antibody, and anti-MOMP serum protects as well as convalescent serum. Vaccination against genital infection with *C. trachomatis* remains a significant challenge. However, future studies using this model of infection to precisely define im-

mune effector mechanisms and to determine how those mechanisms can be elicited locally through vaccination will likely provide essential information for the development of an efficacious vaccine.

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