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# Protection Against *Chlamydia trachomatis* Infection and Upper Genital Tract Pathological Changes by Vaccine-Promoted Neutralizing Antibodies Directed to the VD4 of the Major Outer Membrane Protein

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**The VD4 region from the *Chlamydia trachomatis* major outer membrane protein contains important neutralizing B-cell epitopes of relevance for antibody-mediated protection against genital tract infection. We developed a multivalent vaccine construct based on VD4s and their surrounding constant segments from serovars D, E, and F. Adjuvanted with cationic liposomes, this construct promoted strong immune responses to serovar-specific epitopes, the conserved LNPTIAG epitope and neutralized serovars D, E, and F. Vaccinated mice were protected against challenge, with protection defined as reduced bacterial numbers in vagina and prevention of pathological changes in the upper genital tract. Adoptive transfer of serum and T-cell depletion experiments demonstrated a dominant role for antibodies and CD4<sup>+</sup> T cells in the protective immune response. Integrating a multivalent VD4 construct into the sequence of the major outer membrane protein resulted in a protective and broadly neutralizing vaccine. Our findings emphasize the important role of antibodies in protection against *Chlamydia trachomatis*.**

**Keywords.** broadly neutralizing Ab; VD4; *Chlamydia trachomatis*; vaccine.

*Chlamydia trachomatis* is the causative agent of sexually transmitted disease and eye infections. Worldwide, it is estimated that 100 million persons are infected with genital *C. trachomatis* [1]. The infection can be controlled by antibiotic therapy, but the high prevalence of asymptomatic cases suggests that disease control requires an effective *Chlamydia* vaccine. There is accumulating evidence that an ideal vaccine would need to elicit both cell-mediated and humoral immunity; in particular, the importance of their interplay is becoming increasingly clear [2–5]. If both arms of the immune

system are promoted by a vaccine, the primary role of neutralizing antibodies will be to reduce the initial infectious load; once they are intracellular, remaining bacteria can be targeted by a bactericidal cell-mediated immune (CMI) response.

The majority of preclinical vaccines evaluated mediate protection predominantly through T cells with no neutralizing antibodies [5–8]. Until now there has only been convincing data on the effect of neutralizing antibodies targeted to 3 surface-exposed antigens: Porin B [9], polymorphic membrane protein D, [10] and, in particular, the major outer membrane protein (MOMP). MOMP is highly immunogenic in humans and animals and has been studied in detail as a vaccine candidate (as a natively purified protein vaccine, as a recombinantly expressed protein vaccine and as a DNA-vaccine) (reviewed by Farris and Morrison [5]). Vaccination with MOMP has provided promising but unfortunately also variable, results ascribed mostly to the fact that the recombinant MOMP (rMOMP) immunogen does not mimic the native structure of the protein [11].

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Therefore, although it clearly has potential, full-size MOMP has so far not been a feasible vaccine candidate, and several attempts have been made to construct a vaccine based on selected regions rich in neutralizing target epitopes (eg, the variable domains [VDs]) [12–17]. In particular, the VD4 region has attracted interest, because it was shown to contain the highly conserved species-specific epitope LNPTIAG embedded in the central part of the variable region [18, 19]. Importantly, this conserved epitope in the VD4 region can elicit a broadly cross-reactive immune response able to neutralize multiple serovars; among them, the most prevalent serovar (Sv) D, SvE, SvF, and SvG [13, 20], and is nonconformational, in contrast to other epitopes in MOMP. Previous attempts to construct peptide vaccines representing the VD4 region generated antibodies with some functional capability (measured by means of in vitro neutralization), but this did not translate into in vivo protective efficacy [13, 15–17, 21].

The objective of the current study was to analyze the potential of a vaccine promoted neutralizing antibody response to the MOMP VD4 region. We demonstrate that a multivalent vaccine based on VD4 segments from different serovars raises a high-titered antibody response that neutralizes the most prevalent serovars, reduces early *C. trachomatis* infection in the mouse model, and prevents pathological changes.

## MATERIALS AND METHODS

### Cultivation and Harvesting of *C. trachomatis*

*Chlamydia trachomatis* SvD (UW-3/Cx; American Type Culture Collection [ATCC] VR-885), SvE (BOUR; VR-348B), SvF

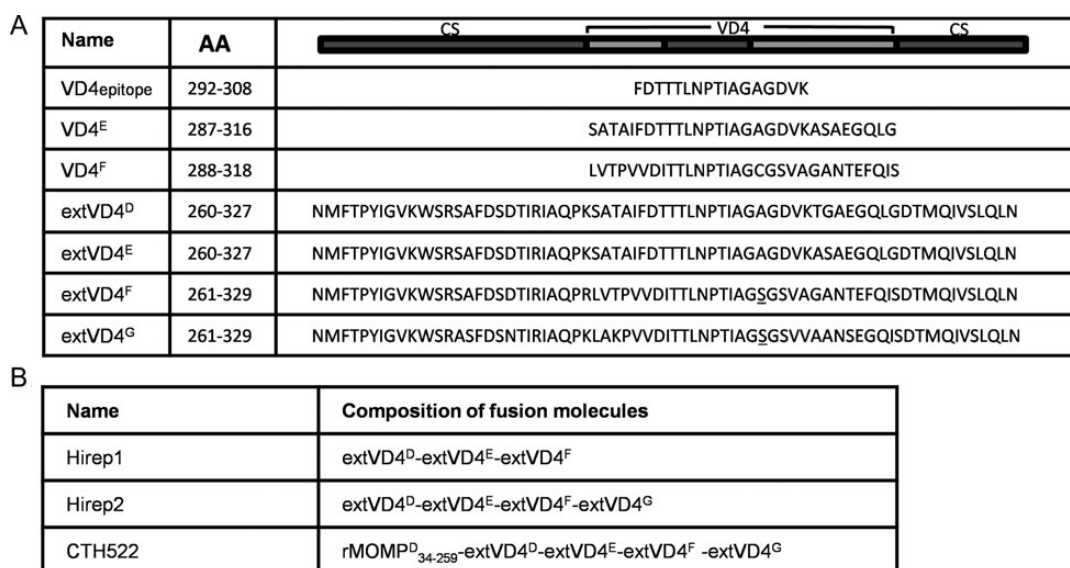
(IC-Cal-3; ATCC VR-346), and SvG (UW-57/Cx) were propagated in Hela 229 cells (ATCC) and purified as described elsewhere [22]. After purification the pellet was resuspended in sucrose-phosphate-glutamate (SPG) buffer and stored at  $-80^{\circ}\text{C}$ . Serovar typing of the bacteria were confirmed by chromosomal DNA extraction, polymerase chain reaction amplification and sequencing of the gene, and flanking regions of *ompA*. The inclusion-forming units (IFUs) of the batches were quantified by titration in McCoy cells, and the protein concentrations were determined by means of bicinchoninic acid protein assay (Pierce).

### Antigen and Fusion Protein Preparation Methods

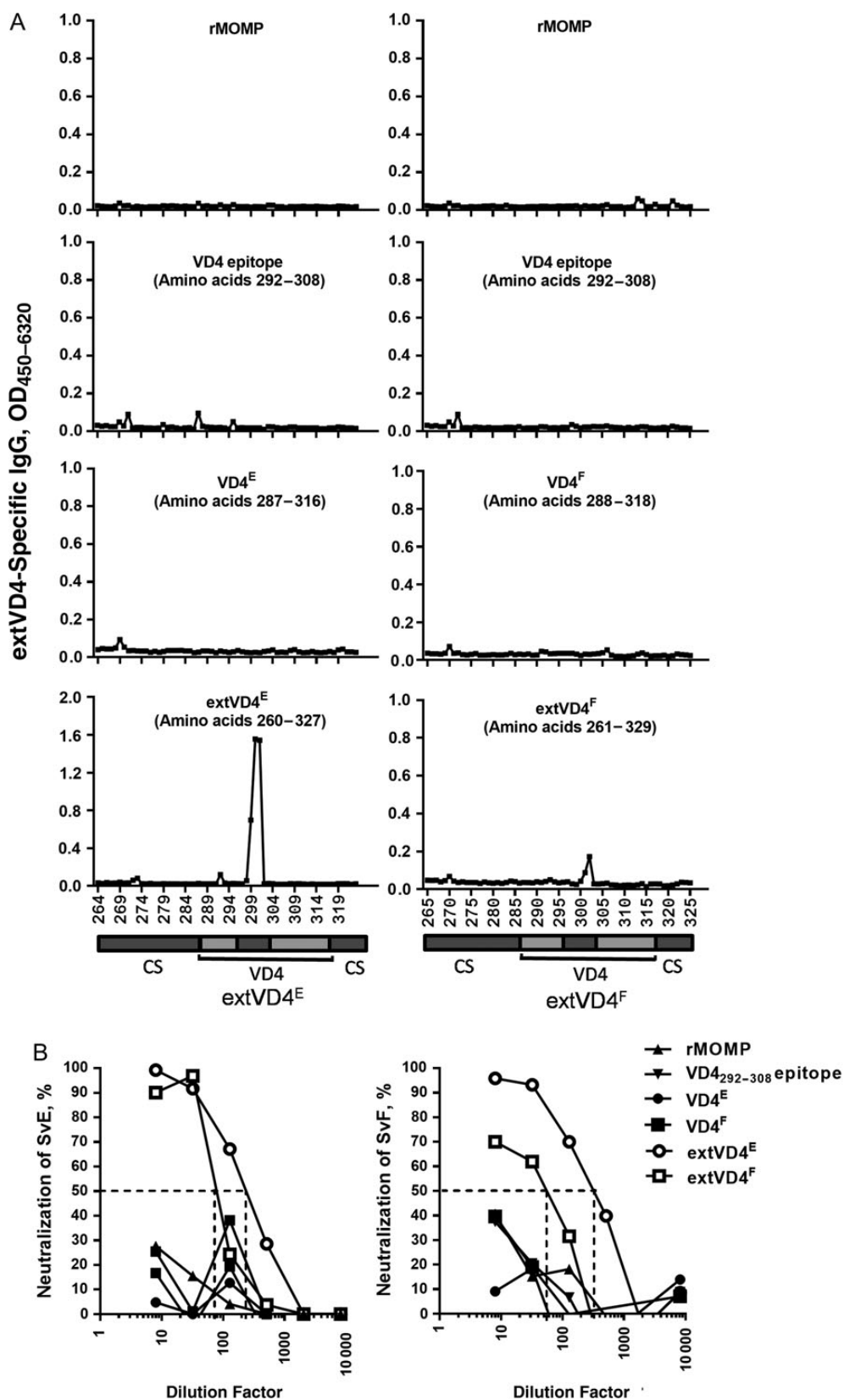
Recombinant proteins (extVD4<sup>E</sup>, extVD4<sup>F</sup>, Hirep1, Hirep2, CTH522 [Figure 1], and rMOMP) were produced as follows. Based on the amino acid sequences (GenBank) with an added N-terminal histidine tag, synthetic DNA constructs were codon optimized for expression in *Escherichia coli*, followed by insertion into the pJexpress 411 vector (DNA2.0). To avoid disulfide bridge formation during recombinant production, all cysteines were exchanged with serines. Purification was done essentially as described elsewhere [23], and VD4<sub>292–308</sub>, VD4<sup>E</sup>, and VD4<sup>F</sup> (Figure 1A) were produced as synthetic peptides (GeneCust). The 9mer biotinylated peptide library was produced by Mimotopes, and the 20mer peptides by GeneCust (Supplementary Table 1).

### Animal Protocol

Female B6C3F1 and C3H/HeN mice, 6–8 weeks of age, were obtained from Harlan Laboratories and C3H/HeJ mice from The



**Figure 1.** Overview of the different VD4-based constructs. *A*, Sequences of the VD4-derived constructs used in the present study. The bar is a schematic overview of the VD4 region; dark gray represents the conserved regions, and light gray the variable regions. In the serovar F and G sequences, the cysteine has been replaced with a serine. *B*, Composition of fusion molecules.



**Figure 2.** Fine specificity of the antibody response after vaccination with constructs representing variable length of the major outer membrane protein (MOMP) VD4 region. *A*, B6C3F1 mice were immunized with the VD4<sub>292–308</sub> peptide ( $n = 4$ ), polypeptides covering the VD4 region (VD4<sup>E</sup> [30 amino acids] and VD4<sup>F</sup> [31 amino acids]) ( $n = 4$ ), or recombinant proteins that included the surrounding constant region (extVD4<sup>E</sup> [67 amino acids] and extVD4<sup>F</sup> [68 amino acids]) ( $n = 4$ ) or full-length recombinant MOMP (rMOMP;  $n = 8$ ). After vaccination, serum samples from immunized mice were pooled and diluted 1:200 and the fine

Jackson Laboratory. The mice were housed under standard environmental conditions and provided standard food and water ad libitum. The use of mice is guided by the regulations set forward by animal protection committees and the Danish Ministry of Justice and in compliance with European Community Directive 86/609.

### Histopathology

Mice were anesthetized and euthanized by cervical dislocation. At necropsy, the entire reproductive tracts were taken out and fixed in formalin. After fixation, the tissue was processed and embedded in paraffin according to standard procedures. Thereafter, 3–4- $\mu$ m sections were cut to include the uterine horns, oviducts, and ovaries in the same section. The sections were mounted on Superfrost glass (Hounisen, Denmark) and stained with hematoxylin-eosin. The sections were assessed blindly by a pathologist.

### Immunization

Mice were immunized 3 times with 14 days between immunizations. The polypeptides were emulsified in cationic adjuvant formulation 1 (CAF01) and administered simultaneously via both subcutaneous and intranasal routes. The vaccines consisted of 5  $\mu$ g of antigen emulsified in 250  $\mu$ g of dimethyldioctadecylammoniumbromide and 50  $\mu$ g of trehalose-6,6-dibehenate (CAF01). Control groups were naive mice or mice receiving CAF01 alone without antigen. For intranasal infection, mice received  $1 \times 10^6$  IFUs of *C. trachomatis* SvD.

### *Chlamydia*-Specific Cellular Responses

Splenocytes were isolated from 4 mice per group 2 weeks after the last vaccination. Single-cell suspensions were prepared from individual spleens. All cell cultures were grown in Nucleon microtiter plates (Nunc), as described elsewhere [8]. The amounts of secreted interferon  $\gamma$  were determined using enzyme-linked immunosorbent assay (ELISA) in readings from 4 individual mice, as described elsewhere [24]. For fluorescence-activated cell sorter analysis, splenocytes were stimulated for 1 hour with 5  $\mu$ g/mL Hirep1 at 37°C and 5% carbon dioxide and subsequently incubated for 5 hours at 37°C with 10  $\mu$ g/mL brefeldin A (Sigma-Aldrich). The intracellular cytokine staining procedure was essentially as described elsewhere [25]. All antibodies were purchased from BD Pharmingen or eBiosciences.

### Measurement of Antibody Levels in Serum and Vaginal Washes

ELISA reactivity against the 9mer overlapping biotinylated peptide panel spanning the extVD4 region of SvE, and SvF (Supplementary Table 1) was investigated. Briefly, ELISA plates were

coated with streptavidin, incubated with biotinylated peptides, blocked with skim-milk powder, and washed, and then the normal ELISA procedure was followed, as described elsewhere [8]. Vaginal wash samples were collected by flushing the vagina with 100  $\mu$ L of sterile phosphate-buffered saline and treated with Bromelain (Sigma). Antigen-specific immunoglobulin (Ig) G1, IgG2a, and IgA were detected, as described elsewhere [8].

### Neutralization Assays

#### *In Vitro* Neutralization Assays

The assay was performed essentially as described elsewhere [26]. Briefly, HaK cells were grown to confluence in 96-well flat-bottom microtiter plates. The *Chlamydia* stocks were diluted and mixed 1:1 with heat-inactivated and diluted serum. The suspension was inoculated onto HaK cells in duplicates and incubated for 24–48 hours. Inclusions were visualized by staining with polyclonal rabbit anti-rCT043 serum, followed by Alexa 488-conjugated goat anti-rabbit immunoglobulin (Life Technologies). Cell staining was done with propidium iodide (Invitrogen). The results were calculated as the percentage reduction in mean IFUs relative to control serum samples. A reduction of  $\geq 50\%$  relative to the control was defined as neutralizing.

#### *In Vivo* Neutralization

Two experiments were performed. First, *C. trachomatis* SvD diluted to  $8 \times 10^7$ /mL in SPG buffer was mixed 1:1 with heat-inactivated and sterile-filtered serum from Hirep1- and rMOMP-vaccinated mice and control mice. After 30 minutes at 37°C, the mice were infected with 10  $\mu$ L of the inoculum (total,  $4 \times 10^5$  bacteria per mouse). They were swabbed on days 3, 7 and 10 after infection, and IFUs were quantified, as described under Vaginal Challenge and Vaginal Chlamydial Load.

#### Passive Transfer of Immune Serum

Serum samples were isolated from 18 (experiment 1) or 24 (experiment 2) mice previously vaccinated 3 times with Hirep1/CAF01 (experiment 1), and either Hirep1/CAF01 or rMOMP/CAF01 (experiment 2). In both experiments, mice vaccinated with CAF01 alone and naive mice were included. The serum samples were heat-inactivated, sterile filtered, and transferred intravenously and intraperitoneally to 6–8 mice. As controls, a pool of mice receiving serum from control mice and naive mice was used. Three days after serum transfer the mice were challenged with *C. trachomatis* SvD and swabbed as described under Vaginal Challenge and Vaginal Chlamydial Load. The results are shown as a pool of the 2 experiments.

*Figure 2 continued.* specificity of the immunoglobulin (Ig) G antibody response was studied using a panel of biotinylated overlapping peptides (9mer with 8-amino acid overlap) representing the extVD4 region from either serovar (Sv) E or SvF. The IgG antibody response to each peptide is depicted at amino acid 5 in each of the 9mer peptides and given as the optical density at 450–620 nm ( $OD_{450-620}$ ). B, In vitro neutralization of SvE and SvF. Serum samples pooled for each group were titrated, mixed with a fixed concentration of bacteria, inoculated onto a HaK cell monolayer, and fixed, and inclusions were counted. Dotted lines represent the reciprocal 50% neutralization titer.

### T-Cell Subpopulation Depletion

Mice were depleted of CD4<sup>+</sup> and/or CD8<sup>+</sup> T cells, essentially as described elsewhere [8]. Two injections of 400 µg purified anti-CD4, anti-CD8 (2 × 200 µg) or a mixture of CD4<sup>+</sup> and CD8<sup>+</sup> antibodies (400 µg of each) were injected at days 7 and 4 before infection, followed by injections of 200 µg at day 1 before and day 2 after infection. The CD4<sup>+</sup> and CD8<sup>+</sup> T-cell depletions were verified by fluorescence-activated cell sorter analysis of peripheral blood mononuclear cells at day 1 after infection using a fluorescein isothiocyanate-conjugated anti-CD4 antibody (clone RM4-4) and a phycoerythrin-conjugated anti-CD8 antibody (clone 53-6; BD Biosciences).

### Vaginal Challenge and Vaginal Chlamydial Load

At 10 and 3 days before *C. trachomatis* SvD challenge, the estrus 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 4–8 × 10<sup>5</sup> IFUs of *C. trachomatis* SvD or 1 × 10<sup>6</sup> IFUs of *C. trachomatis* SvF. Vaginal swab samples were obtained at 3, 7, and 10 days after infection. Infectious load was assessed as described elsewhere [8]. Inclusions were enumerated by fluorescence microscopy. Culture-negative mice were assigned the lower cutoff of 4 IFUs per mouse.

### Statistical Analysis

GraphPad Prism 6 software was used for data handling, analysis, and graphic representation. Statistical analysis was performed using the Kruskal–Wallis test followed by the Dunn's post test or the Mann–Whitney test.

## RESULTS

### Enhancing the Magnitude of the Immune Response to the LNPTIAG Region by Optimizing the Length of the VD4 Region

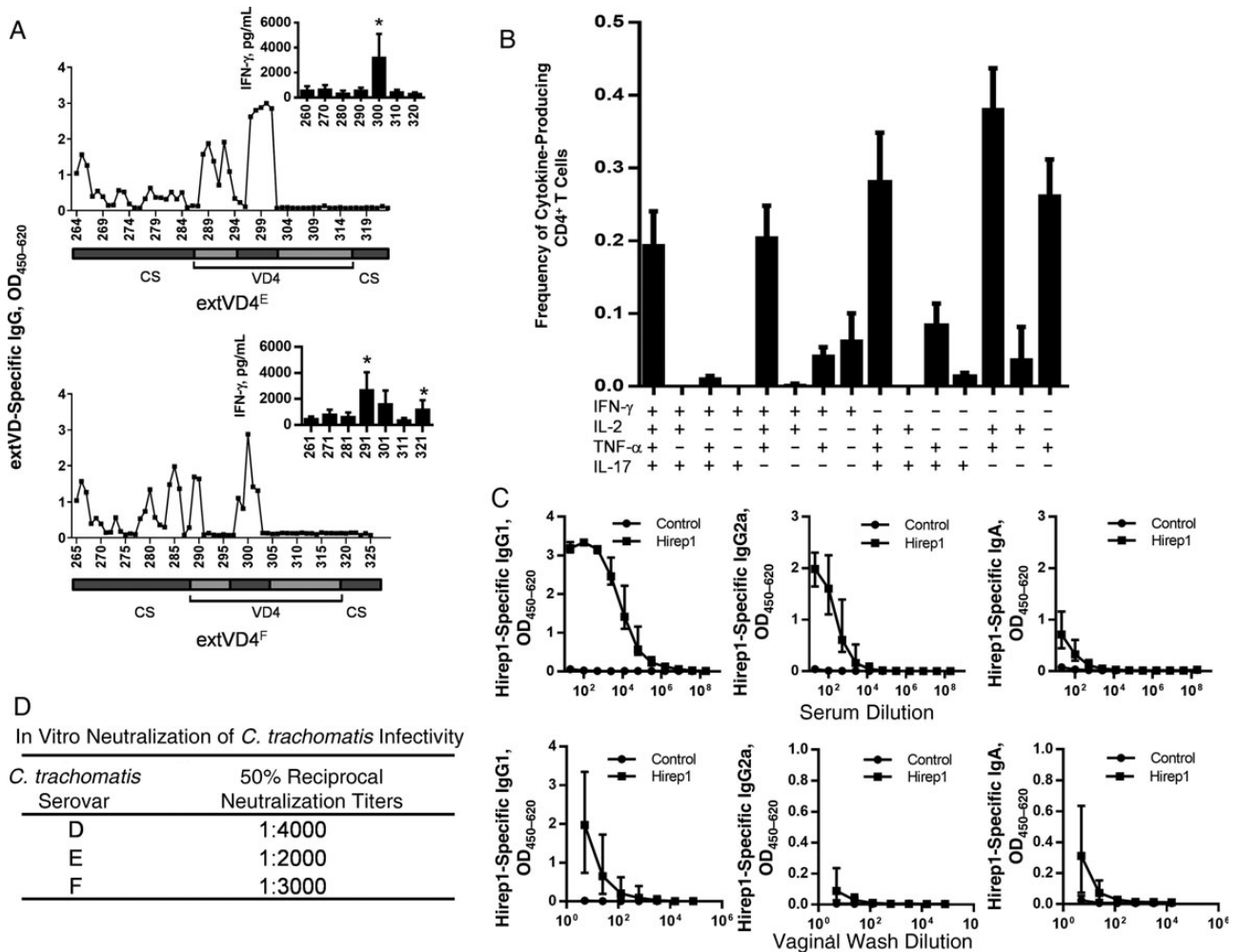
The VD4 region and its surrounding membrane anchor are reported to contain numerous T- and B-cell epitopes [27, 28]. We therefore analyzed the influence of including in the immunogens different parts of this surrounding region on the specificity and magnitude of the antibody response to the VD4 epitopes. We tested peptides and proteins of increasing length and compared the immune responses. Peptides representing the neutralizing LNPTIAG epitopes (VD4<sub>292–308</sub>: 17 amino acids) and the complete VD4 region of SvE (VD4<sup>E</sup>: 30 amino acids) and SvF (VD4<sup>F</sup>: 31 amino acids) were compared with recombinant proteins representing extended VD4 regions (extVD4<sup>E</sup>: 67 amino acids and extVD4<sup>F</sup>: 68 amino acids), which include the conserved membrane anchoring residues (Figure 1A). Responses were compared with responses to immunization with full-length rMOMP. The antigens were administered in CAF01, a liposome adjuvant, demonstrated elsewhere to induce a strong immune response that balances the humoral and CMI response [29].

Postvaccination antibody responses were analyzed against the VD4 region, using a biotinylated peptide library with panels of overlapping peptides (9mer with 8–amino acid overlap) (Supplementary Table 1). No specific antibodies were induced by rMOMP, the VD4 epitope, or the peptide covering the complete VD4 region, whereas the extVD4<sup>E</sup> region (67 amino acids) induced a strong response that mapped to the LNPTIAG region (Figure 2A). The extVD4<sup>F</sup> sequence also induced a response to LNPTIAG but at a much lower level than the SvE construct. The response against the LNPTIAG region correlated with the ability to neutralize a *C. trachomatis* SvE and SvF infection in vitro (Figure 2B). No neutralizing antibody response was promoted by rMOMP, the peptides representing the LNPTIAG epitope or the VD4 region, whereas the extended constructs, especially the extVD4<sup>E</sup>, induced high titers of neutralizing antibodies.

### Broadly Neutralizing Antibody Responses and Protection Against Vaginal Infection and Upper Genital Tract Pathological Changes Promoted by a Fusion of the VD4 Region From Different Serovars

To investigate whether it is possible to expand the recognition pattern and include VD4 regions from multiple serovars, we designed a molecule that incorporated the extended VD4 region from SvD, SvE, and SvF into a single multivalent construct (heterologous immuno-repeat 1; Hirep1). This construct was very immunogenic and induced a strong and diverse response directed to numerous B- and T-cell epitopes, in both the conserved and specific regions of VD4 from SvE and SvF (Figure 3A and Supplementary Table 1). In addition to interferon  $\gamma$ , the T cells promoted by this construct expressed interleukin 2, tumor necrosis factor  $\alpha$ , and interleukin 17 in various combinations, and polyfunctional T cells that expressed more than one cytokine dominated the overall response. The antibody response was characterized by IgG1/IgG2a antibodies in the serum samples and relatively high levels of IgG1 and IgA in the vaginal wash samples (Figure 3B and C). The antibody response to Hirep1 efficiently neutralized SvD, SvE, and SvF, which were represented in the construct (Figure 3D).

We continued by investigating and characterizing the Hirep1 protective efficacy in a genital *C. trachomatis* SvD challenge model. We evaluated the influence of the infectious dose and the strain of mice to allow detection of neutralizing antibodies without excessive innate immune activation (Supplementary Figure 1). The mouse strain B6C3F1 (C57Bl/6J × C3H/HeN) and a dose of 4–8 × 10<sup>5</sup> IFUs per mouse were selected, because the relatively low inoculum provided a protective window for the monitoring of adaptive immunity found in mice with the C3H background (Supplementary Figure 1), combined with a strong response to the chlamydial MOMP VDs reported elsewhere for mice with the C57Bl/6J background [16]. Mice vaccinated with Hirep1/CAF01 had 100–200-fold reduced chlamydial shedding at days 3 and 10 after infection, compared with



**Figure 3.** Immune responses induced with a multivalent vaccine based on extVD4 from serovar (Sv) D, SvE, and SvF (Hirep1). *A*, Serum samples from 16 mice per group were pooled, and the fine specificity of antibody responses analyzed using the biotinylated peptide library. Insets show VD4-specific interferon (IFN)  $\gamma$  responses; bars represent mean and standard errors of the mean for 4 mice. \* $P < .05$  (Mann-Whitney test for comparison against peptide responses in naive mice). *B*, Splenocytes from 4 mice were isolated, stimulated with Hirep1 and analyzed with intracellular flow cytometry. The frequency of CD4<sup>+</sup> T cells producing any combination of IFN- $\gamma$ , tumor necrosis factor (TNF)  $\alpha$ , interleukin 2 (IL-2), or interleukin 17 (IL-17) was analyzed using Boolean gating. *C*, Serum samples ( $n = 16-18$ ) and vaginal wash samples ( $n = 16$ ) were serially diluted and added to Hirep1-coated plates and antigen-specific IgG1, IgG2a, and IgA were analyzed by means of enzyme-linked immunosorbent assay. Individual points represent median optical density values (with interquartile range) at each titration step. *D*, In vitro 50% reciprocal neutralization titers to SvD, SvE, and SvF (pool of serum from 16 mice) (for details, see Figure 2). Abbreviations: Ig, immunoglobulin; OD<sub>450-620</sub>, optical density at 450–620 nm.

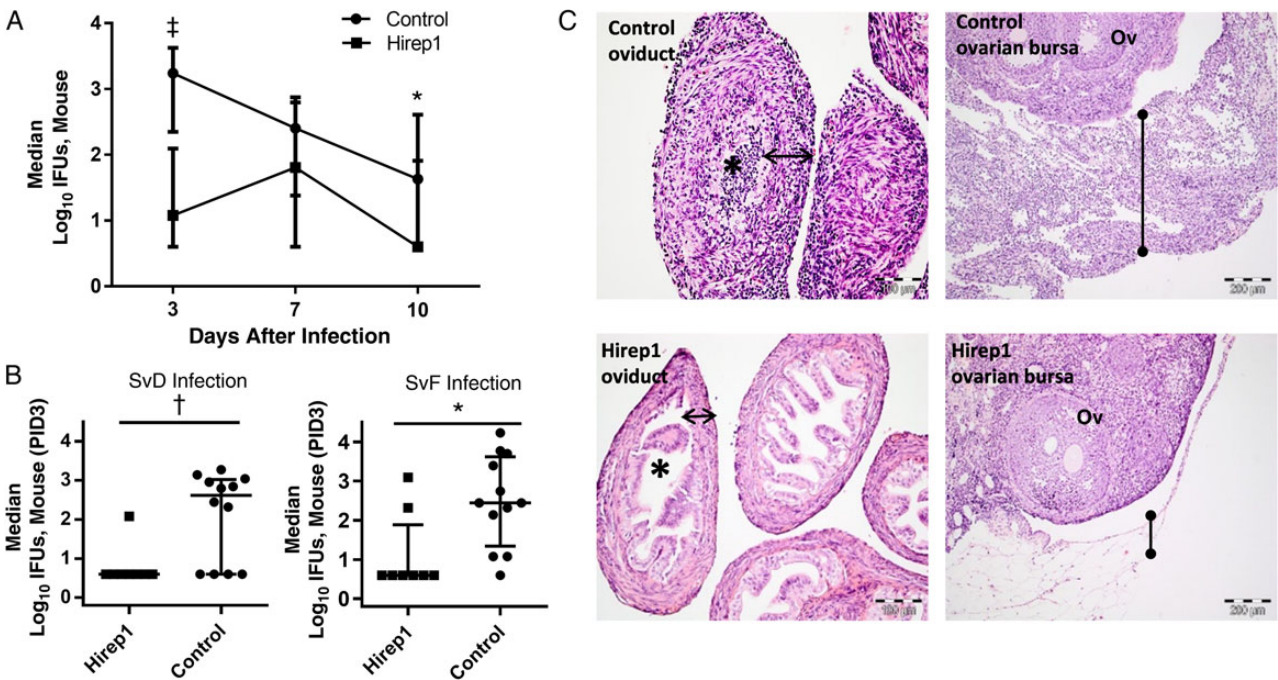
the control mice (Figure 4A). The same tendency was seen at day 7, although at this point the protection did not reach statistical significance compared with unvaccinated controls. The early protection at day 3 was found against both SvD and SvF genital challenge (Figure 4B).

The reproductive tracts were histopathologically assessed. Infected unvaccinated mice had substantial oviduct pathology, whereas Hirep1-vaccinated mice had no pathological changes. The pathological changes in the unvaccinated mice was characterized by moderate to severe lymphocyte infiltration of the oviduct wall with dense filling of debris and neutrophils in the lumen of the oviduct (pyosalpinx) (Figure 4C, top left panel). In the

affected oviducts, degenerative changes were found in the epithelium, with blunting of villi and interspersed leucocytes. The mice also had a very pronounced lymphocyte infiltration in the mesosalpinx and ovarian bursa (Figure 4C, top right panel). None of these changes were seen in Hirep1-vaccinated mice. Mild to moderate lymphocyte accumulation and glandular cysts were observed in the uterine horns from mice in both groups.

#### Hirep1 Protection is Mediated Through Early Antibody and CMI responses

To further investigate the relative role of neutralizing antibodies and T-cell responses in the protective response observed in



**Figure 4.** Hirep1-induced protection and pathological changes after genital *Chlamydia trachomatis* challenge. *A*, B6C3F1 mice were vaccinated with Hirep1 and 6 weeks later challenged with  $4 \times 10^5$  inclusion-forming units (IFUs) of *C. trachomatis* serovar (Sv) D. Data are presented as log<sub>10</sub> IFUs, and each point represents the median number (and interquartile range [IQR]) recovered from vaginal swab samples at days 3, 7, and 10 after infection, from a pool of 4 identically designed individual experiments (Hirep1,  $n = 36$ ; controls,  $n = 40$ ).  $*P < .05$ ;  $\ddagger P < .001$ . Mann–Whitney test. *B*, Hirep1-vaccinated mice were challenged with *C. trachomatis* SvD and SvF bacteria. The IFU values are displayed as scattered plots with the medians and IQRs depicted ( $n = 8$ –16 per group). Mann–Whitney test was used for comparison among groups.  $*P < .05$ ;  $\ddagger P < .01$ . *C*, Histological sections of oviducts and ovaries from a naive mouse (*upper panels*) and a Hirep1-vaccinated mouse (*lower panels*). The walls of the oviducts are denoted with double-headed arrows, the oviduct lumens with asterisks, and the ovarian bursae with line segments. Abbreviation: Ov, ovary.

the vaccinated animals, a group of mice was vaccinated with Hirep1, rMOMP, or adjuvant alone. Serum samples from vaccinated and control mice were isolated, pooled within the group, and adoptively transferred into naive recipient mice, followed by challenge 3 days later. The transfer of serum from Hirep1-immunized animals resulted in a 100-fold reduction in IFUs, whereas serum from animals immunized with rMOMP showed no protective effect (Figure 5A). The role of antibodies was also confirmed by in vivo neutralizing experiments, in which *C. trachomatis* SvD was preincubated with serum samples from vaccinated and control mice before challenge. Serum samples from Hirep1-vaccinated mice almost completely ablated the ability of *C. trachomatis* to establish a genital tract infection, whereas serum samples from rMOMP-immunized mice had no influence on bacterial numbers (Figure 5B).

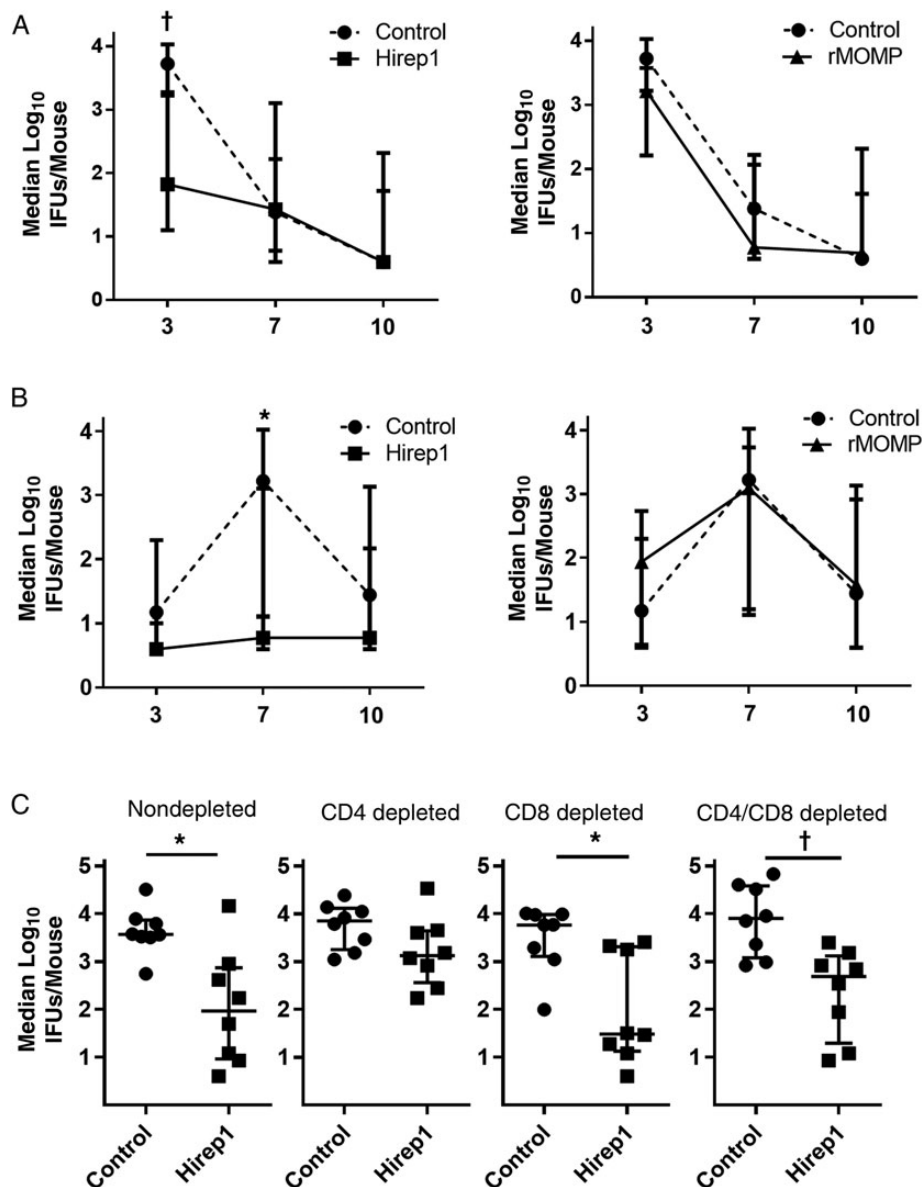
To further characterize the effector mechanism responsible for the early protection (day 3), Hirep1-vaccinated mice were depleted of CD4<sup>+</sup> and/or CD8<sup>+</sup> T cells before challenge (4 injections, at days 7, 4, and 1 before and day 2 after infection), and protection was assessed at day 3 after infection. Hirep1 vaccination reduced the bacterial numbers almost 100-fold. Depleting both T-cell subsets still reduced the number of bacteria approxi-

mately 10-fold compared with controls. The CD8<sup>+</sup> T-cell depletion had no influence on protection, but CD4<sup>+</sup> T-cell depletion reduced the protection to a level comparable to that in the CD4<sup>+</sup>/CD8<sup>+</sup>-depleted group. Taken together, these observations confirm the importance of antibodies in the early protection promoted by Hirep1 vaccination but suggest that the CD4<sup>+</sup> T-cell response also plays a role (Figure 5C).

#### VD4 Specific Broadly Neutralizing Antibodies Obtained by Integrating a Multivalent VD4 Construct Into rMOMP

We continued by investigating whether the powerful neutralizing ability of Hirep1 could be transferred to the MOMP molecule by integrating the extVD4s from SvD, SvE, SvF, and SvG into the sequence of rMOMP. We hypothesized that by combining the neutralizing antibodies against VD4 with the numerous protective T-cell epitopes localized in the remaining part of rMOMP, we would improve protection. First we designed a construct that in addition to SvD, SvE, and SvF included also the extVD4 region from SvG (Hirep2; Figure 1B). Compared with Hirep1, this construct induced similar levels of protection and neutralizing antibody titers (Supplementary Figure 2). After confirmation of its biological activity, Hirep2 was integrated into a version of





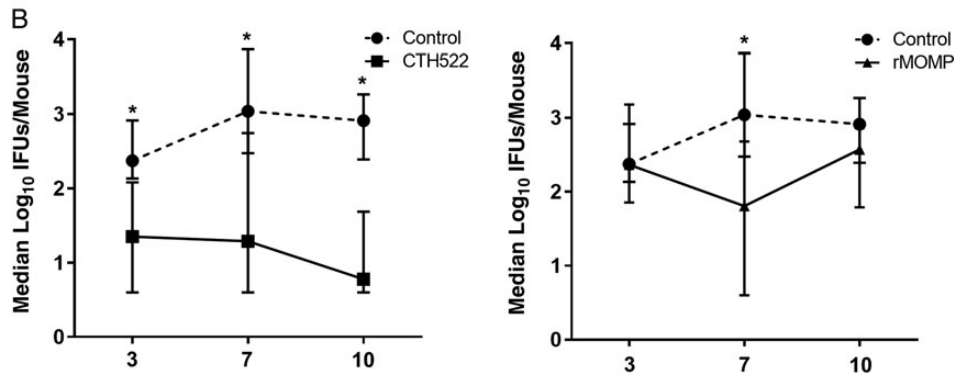
**Figure 5.** The role of specific antibodies in protection promoted by Hirep1 and recombinant major outer membrane protein (rMOMP). *A*, Adoptive transfer of protection against *Chlamydia trachomatis* serovar (Sv) D with Hirep1- and rMOMP-specific serum. Hirep1- and rMOMP vaccinated mice were bled, and serum samples were isolated, heat inactivated, sterile filtered, and transferred to naive recipient mice 3 days before infection. Three days after transfer, mice were challenged with  $4 \times 10^5$  inclusion-forming units (IFUs) of *C. trachomatis* SvD. Data are presented as  $\log_{10}$  IFUs, and each point represents the median number (and interquartile range [IQR]) recovered from vaginal swab samples at days 3, 7, and 10 after infection (Hirep1,  $n = 16$ ; rMOMP,  $n = 6$ ; controls,  $n = 31$ ). Results represent a pool of 2 individual experiments. The Dunn's multiple comparison test was used for comparisons among groups.  $\dagger P < .01$ . *B*, In vivo neutralization of SvD with Hirep1 and rMOMP-specific serum. *C. trachomatis* SvD was incubated with heat-inactivated serum samples from vaccinated and control mice before infection ( $4 \times 10^5$  IFUs per mouse). In vivo neutralization was assessed by means of *Chlamydia* culture at days 3, 7, and 10 after challenge. Data are presented as  $\log_{10}$  IFUs, and each point represents the median number (and IQR). Results represent a pool of 2 individual experiments. The Dunn's multiple comparison test was used for comparison among groups  $*P < .05$ . *C*, Mice were depleted of CD4<sup>+</sup> and CD8<sup>+</sup> T cells by injection of monoclonal antibodies (anti-mouse CD4 [clone GK1.5] and/or anti-mouse CD8 [clones YTS156 and YTS169]) at days 7, 5, and 1 before and day 2 after infection. Protection was assessed at day 3 after infection. Each point represents the number of IFUs recovered from vaginal swab samples, and lines represent the median and IQR ( $n = 8$ ). The Mann-Whitney test was used for comparisons among groups.  $*P < .05$ ;  $\dagger P < .01$ .

rMOMP and truncated at the cysteine-rich N-terminus to facilitate recombinant production (rMOMP<sub>34-259</sub>-Hirep2: CTH522) (for the design, see Figure 1). CTH522 was compared with rMOMP in a genital SvD challenge experiment. Compared with

vaccination with rMOMP, CTH522 induced high levels of neutralizing antibodies against the 4 serovars SvD, SvE, SvF, and SvG (Figure 6A) and provided significant protection at both early and later time points (Figure 6B).

A

In Vitro Neutralization of <i>C. trachomatis</i> Infectivity		
<i>C. trachomatis</i> Serovar	50% Reciprocal Neutralization Titers	
	CTH522	rMOMP
D	1:1000	<8
E	1:1000	<8
F	1:2500	<32
G	1: 360	<8



**Figure 6.** In vitro neutralization titers and protection induced by vaccination. B6C3F1 mice were vaccinated with CTH522 (n = 16) or recombinant major outer membrane protein (rMOMP; n = 16) (controls, n = 36). A, In vitro neutralization of serovars D, E, F, and G (for details, see Figure 2). B, Six weeks later, the mice were challenged with *Chlamydia trachomatis* serovar D. Values represent median numbers of inclusion-forming units (IFUs) (with interquartile ranges) recovered from vaginal swab samples at days 3, 7, and 10 after infection. The Dunn's multiple comparison test was used for comparison among groups. \*P < .001.

## DISCUSSION

This study was focused on the vaccine potential of a neutralizing antibody response against *C. trachomatis* infection. Our first aim was to optimize an antigen molecule based on the VD4 region by increasing the size of the variable VD4 segment (containing the neutralizing linear LNPTIAG epitope), to a molecule that includes T- and B-cell epitopes from the conserved segments (extVD4s) [27, 28]. We showed that recombinant full-length MOMP does not target the VD4 region and that antibodies are non-neutralizing, in contrast to our optimized constructs that induce robust levels of neutralizing antibodies. This is in agreement with findings in a seminal study by Su and Caldwell [13], which demonstrated that a 17 amino acids VD4-derived peptide covering the LNPTIAG epitope needs T-cell help, which could be provided by fusing with a T-cell epitope. This important observation stimulated extensive research wherein peptides from the VD4 region were used either alone, fused to other regions such as VD1, or mixed with T-cell epitopes to potentiate the antibody response [13, 15–17, 21, 30]. All these constructs generated antibodies with some neutralizing ability, but, importantly they did not translate into in vivo protective efficacy against genital chlamydial challenge [13, 17].

Compared with these classic observations, our approach gives rise to a response that differ in both magnitude and breadth of the antibody response and by the fact that a strong T-cell response is promoted in addition to the humoral response. We make the important observation that the antibody response is potentiated and expanded by combining closely related but different VD4 inserts into a single multivalent construct. The multivalent Hirep constructs based on VD4s from SvD, SvE, and SvF promoted a strong antibody response that both amplified the response to the LNPTIAG region shared by the inserts and also increased the breadth of the response to include recognition of several serovar-specific VD4 antibody epitopes, some of which have been described elsewhere [20, 21]. Antibodies have received renewed focus as mediators of protective immunity against *C. trachomatis* infection. Although knockout and depletion models have failed to demonstrate a significant role of antibodies in the natural immune response during primary infection [31], their involvement in protection against reinfection and as part of an adaptive acquired immune response is becoming increasingly clear [32–35].

Our findings demonstrate that when a high-titered neutralizing antibody response is obtained, immune serum samples can adoptively protect recipient mice against a primary challenge. To our knowledge, this is the first time that an in vitro

neutralization effect has been translated into in vivo protection by vaccination, highlighting the importance of antibody-mediated protection against this pathogen. To promote both systemic and mucosal antibody responses, we chose a vaccination protocol wherein the mice were simultaneously vaccinated subcutaneously and intranasally. Comparing this vaccination strategy with the subcutaneous route, we observed increased levels of local IgA in vaginal secretions, but not a statistically significant difference between the 2 protocols, when we assessed the level of neutralizing serum antibodies or protection against genital challenge (results not shown). This, together with the observation that adoptive transfer of antibodies can induce early protection against challenge, suggests that systemic neutralizing antibody is sufficient for genital tract protection in our mouse model.

Importantly, the Hirep1 vaccine has a pronounced impact on controlling the ascending infection and completely protects infected mice against upper genital tract pathology (Figure 4). This finding is in agreement with the classic observations that adoptive transfer of a large amount of MOMP-specific monoclonal antibody can reduce pathology [36] and suggests that antibody elimination of the infectious inoculum reduces the bacterial numbers. Pathology as a readout has been the subject of discussion in the mouse model using human strains for challenge. A concern is that in the mouse model, *C. trachomatis* infection may be eliminated predominantly by innate mechanisms before the bacteria ascend to the upper part of the genital tract, preventing a meaningful readout of adaptive immune responses and pathological changes [37].

In the present study we have therefore tested the influence of the inoculating dose and strain of mice (Supplementary Figure 1), and have used the B6C3F1 strain and a dose of  $4-8 \times 10^5$  IFUs per mouse throughout our studies to maximize the “protective window” and minimize the contribution of innate immune activation [38]. This model allows the monitoring of early vaccine-promoted adaptive immunity, measured as a reduction in both bacterial numbers and upper genital tract pathological changes. Hirep1-vaccinated mice had significantly less neutrophil infiltration in their genital tracts than control mice, again supporting the notion that adaptive immunity and not innate inflammation controls infection in vaccinated mice. Clearly, the mouse model using *Chlamydia muridarum* infection is a preferable model for evaluating *Chlamydia* pathogenicity in the upper genital tract, but for vaccine studies this strain suffer from the major limitation that many of the antigens differ from human serovars; for example, the neutralizing epitope that is the subject of the present study is lacking.

Our depletion study suggests an important synergistic role of CD4<sup>+</sup> T cells promoted by the CAF01 liposomal adjuvant system, in addition to the neutralizing antibody response. The CMI response seen after CAF01 immunization is dominated by multifunctional Th1/Th17 cells promoted by the C-type

lectin incorporated into the CAF liposomes, and this phenotype seems independent of the choice of antigen [25, 39–41]. The CD4<sup>+</sup> T cells may have a direct effector function, as reported for MOMP-specific Th1 cells [6], or may also accelerate the early mucosal immune responses and IgA secretion [42]. The Th17 response promoted by CAF01 has been suggested to be important for the *C. trachomatis* protection promoted by vaccines based on this adjuvant [41], in support of the need for a vaccine and an adjuvant, that promotes both CMI and humoral responses to a *C. trachomatis* vaccine.

Although clearly very susceptible to conformational changes, the extVD4 from SvD, SvE, SvF, and SvG could be integrated into the sequence of rMOMP (CTH522). This molecule benefits from both the VD4 neutralizing epitopes and numerous T-cell epitopes in MOMP [27, 28, 43–45]. CTH522 neutralized SvD, SvE, SvF, and SvG, which represent up to 90% of the human *C. trachomatis* prevalence [46–50], suggesting that a vaccine based on this method could have a tremendous impact on the global *C. trachomatis* epidemic.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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