Human Vaccines & Immunotherapeutics

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/khvi20

# Intranasal delivery of *Salmonella* OMVs decorated with *Chlamydia trachomatis* antigens induces specific local and systemic immune responses

**Dung T. Huynh, Emanuele Nolfi, Lobna Medfai, Peter van Ulsen, Wouter S. P. Jong, Alice J. A. M. Sijts & Joen Luirink**

**To cite this article:** Dung T. Huynh, Emanuele Nolfi, Lobna Medfai, Peter van Ulsen, Wouter S. P. Jong, Alice J. A. M. Sijts & Joen Luirink (2024) Intranasal delivery of *Salmonella* OMVs decorated with *Chlamydia trachomatis* antigens induces specific local and systemic immune responses, Human Vaccines & Immunotherapeutics, 20:1, 2330768, DOI: 10.1080/21645515.2024.2330768

**To link to this article:** https://doi.org/10.1080/21645515.2024.2330768

© 2024 The Author(s). Published with license by Taylor & Francis Group, LLC.

View supplementary material

Published online: 22 Mar 2024.

Submit your article to this journal

Article views: 709

View related articles

View Crossmark data

Full Terms & Conditions of access and use can be found at https://www.tandfonline.com/action/journalInformation?journalCode=khvi20

HUMAN VACCINES & IMMUNOTHERAPEUTICS
2024, VOL. 20, NO. 1, 2330768
https://doi.org/10.1080/21645515.2024.2330768

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

OPEN ACCESS Check for updates

# Intranasal delivery of *Salmonella* OMVs decorated with *Chlamydia trachomatis* antigens induces specific local and systemic immune responses

Dung T. Huynh[a,b*], Emanuele Nolfi[c*], Lobna Medfai[c], Peter van Ulsen[b], Wouter S. P. Jong[a], Alice J. A. M. Sijts[c], and Joen Luirink[a,b]

[a]R&D department, Abera Bioscience AB, Uppsala, Sweden; [b]Group of Molecular Microbiology, Amsterdam Institute for Life and Environment (A-LIFE), Vrije Universiteit Amsterdam, Amsterdam, The Netherlands; [c]Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

**ABSTRACT**

*Chlamydia trachomatis* is an obligate intracellular pathogen responsible for the most prevalent bacterial sexually transmitted disease globally. The high prevalence of chlamydial infections underscores the urgent need for licensed and effective vaccines to prevent transmission in populations. Bacterial outer membrane vesicles (OMVs) have emerged as promising mucosal vaccine carriers due to their inherent adjuvant properties and the ability to display heterologous antigens. In this proof-of-concept study, we evaluated the immunogenicity of *Salmonella* OMVs decorated with *C. trachomatis* MOMP-derived CTH522 or HtrA antigens in mice. Following a prime-boost intranasal vaccination approach, two OMV-based *C. trachomatis* vaccines elicited significant humoral responses specific to the antigens in both systemic and vaginal compartments. Furthermore, we demonstrated strong antigen-specific IFN-γ and IL17a responses in splenocytes and cervical lymph node cells of vaccinated mice, indicating $CD4^+$ Th1 and Th17 biased immune responses. Notably, the OMV-CTH522 vaccine also induced the production of spleen-derived $CD8^+$ T cells expressing IFN-γ. In conclusion, these results highlight the potential of OMV-based *C. trachomatis* vaccines for successful use in future challenge studies and demonstrate the suitability of our modular OMV platform for intranasal vaccine applications.

**ARTICLE HISTORY**
Received 3 August 2023
Revised 5 March 2024
Accepted 12 March 2024

**KEYWORDS**
Chlamydia; *Chlamydia trachomatis*; *Salmonella*; OMV; vesicle; mucosal vaccine; sexually transmitted disease; nanoparticle; extracellular vesicle

## Introduction

*Chlamydia trachomatis* is the most prevalent sexually transmitted bacterium worldwide, with an estimated global prevalence of ~4%.[1] It can cause cervicitis and urethritis and, in the long term, chronic abdominal pain and infertility. However, *C. trachomatis* infections often remain asymptomatic, making intervention strategies challenging in addressing transmission in populations.[2] *C. trachomatis* has a complex lifecycle that involves switching between infectious elementary bodies (EBs) and replicating reticulate bodies (RBs). The metabolically inactive EBs convert into RBs upon endocytosis, allowing replication and differentiation within host cells. Eventually, the mature EBs are released and can infect neighboring cells.[3] Under stressful conditions such as exposure to IFN-γ, antibiotics, or nutrient deprivation, *C. trachomatis* can enter a persistence phase that enables its survival in the harsh intracellular environment.[4] Current treatment for *Chlamydia* primarily relies on antibiotics, but the partly intracellular lifestyle and its ability to enter a persistence phase have spurred widespread efforts to develop a broadly protective vaccine.[5]

The specific immune responses that provide protection against *C. trachomatis* through vaccination are not well-defined. Nonetheless, numerous (pre)clinical studies on whole-cell-based or recombinant subunit vaccines for chlamydia have emphasized the significance of neutralizing antibodies and $CD4^+$ Th1 cells producing IFN-γ.[6] It is important to note that *C. trachomatis* is a pathogen that primarily resides in the mucosa of the upper and lower genital tract, evading host defenses.[7] Mucosal administration routes for chlamydial vaccines have demonstrated the ability to generate local immunity capable of preventing both transmission and disease.[5] Intranasal administration is considered highly desirable due to its ease of use and ability to trigger systemic and local immune responses.[8] However, the development of intranasal vaccines has faced challenges owing to the lack of suitable and safe adjuvants and the complex assessment of mucosal immune responses that contribute to protection, particularly at distant sites.[8,9]

Recently, bacterial outer membrane vesicles (OMVs) have emerged as an immunostimulatory carrier for intranasal delivery.[10–12] OMVs are nanoparticles that naturally shed from the outer membrane of Gram-negative bacteria. They contain pathogen-associated molecular patterns which can bind to Toll-like receptors present on host cells, consequently activating innate and adaptive immune responses.[13,14]

**CONTACT** Joen Luirink s.luirink@vu.nl Group of Molecular Microbiology, Amsterdam Institute for Life and Environment (A-LIFE), Vrije Universiteit Amsterdam, Amsterdam, The Netherlands; Alice J. A. M. Sijts e.j.a.m.sijts@uu.nl Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands.

*These authors have contributed equally to this work.

Supplemental data for this article can be accessed on the publisher's website at https://doi.org/10.1080/21645515.2024.2330768.

© 2024 The Author(s). Published with license by Taylor & Francis Group, LLC.
This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial License (http://creativecommons.org/licenses/by-nc/4.0/), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited. The terms on which this article has been published allow the posting of the Accepted Manuscript in a repository by the author(s) or with their consent.

Previously, we engineered the "display" *E. coli* hemoglobin protease (HbpD) autotransporter to present multiple heterologous antigens at the surface of LPS-detoxified *Salmonella* Typhimurium OMVs.[15,16] In essence, domains that protrude from the core β-helix structure of HbpD were replaced by antigenic sequences through translational fusion. In previous proof-of-concept studies, we have shown that epitopes of the model antigen ovalbumin (OVA) presented in this context, induced maturation of professional dendritic cells (DCs) allowing for a tailored induction of immunity.[17] Furthermore, intranasal immunization with recombinant OMVs decorated with fragments of the *Streptococcus pneumoniae* PspA antigen was shown to offer protection against colonization,[16,18] while those displaying Skp antigens protected mice against enterotoxigenic *E. coli* (ETEC) challenge in mice.[19] Yet, this "display" technology in OMVs was not always compatible with the expression of large, structurally complex or post-translationally modified antigens.[20] To address this issue, we now use the SpyTag/SpyCatcher (SpT/SpC) protein ligation system to link separately expressed and purified antigens to HbpD at the OMV surface.[21] This alternative approach was recently used to couple the receptor-binding domain of the SARS-CoV-2 Spike protein, purified from human HEK cells, to OMV-HbpD, generating a nasal vaccine that was strongly immunogenic and protective against SARS-CoV-2 challenge in a golden hamster model.[12]

Here, we continue exploring the latter technology to produce and test OMVs decorated with purified *C. trachomatis* antigens. Major outer membrane protein (MOMP)-derivative CTH522[22] and a proteolytically inactive variant of HtrA were selected as antigens for the coupling strategy. MOMP accounts for ~60% of the total outer membrane mass and comprises serotype-specific extracellular variable domains (VDs) that contain confirmed B-cell epitopes and the core β-barrel with multiple T-cell epitopes.[23,24] Recombinant CTH522 consists of a large part of mature MOMP (residues 34–259) and the VD4 of four genital serovars (SvD-G) in tandem.[22] This engineered CTH522 fusion protein induced strong vaccine-specific systemic and mucosal immune responses after parenteral priming (3×) followed by intranasal boosting (2×) in the first-in-human trial conducted recently.[25] HtrA is a periplasmic serine protease/chaperone that plays an important role in the quality control of cell envelope biogenesis in Gram-negative bacteria.[26] Of note, *C. trachomatis* HtrA was also detected at the surface of EBs and RBs and secreted in the supernatant either as soluble protein or in OMVs and could even be detected in the cytosolic fraction of host cells.[26–29] Chlamydial HtrA expression is upregulated in the persistence phase and serves as a clinical marker for detection of infection.[4,30,31] The endoprotease activity of HtrA can be inactivated by altering the active site serine (S) at residue 247 to alanine (A), generating a proteolytically inactive variant that is safe for vaccine applications.[32]

We tested the immunogenicity of recombinant *Salmonella* OMVs decorated with either CTH522 or HtrA upon intranasal application in mice. Both OMV-based *C. trachomatis* vaccines proved to be innocuous and strongly immunogenic. The vaccines elicited robust responses from helper $CD4^+$ Th1/Th17 cells and induced significant humoral immune responses in both systemic and vaginal compartments. Importantly, the OMV-CTH522 vaccine also triggered a cytotoxic $CD8^+$ T cell response. These findings suggest that both recombinant OMV-based *C. trachomatis* vaccines hold promise for future challenge studies, either as stand-alone vaccines or in combination with prime-boost immunization strategies.

## Materials and methods

### Bacterial strains and growth

*E. coli* BL21(DE3)-derivative ClearColi (DE3)[33] was used for protein expression. Cells were grown at 37°C in lysogeny broth (LB; 10 g/liter tryptone, 5 g/liter yeast extract, and 10 g/liter NaCl), supplemented with 1 mM $MgCl_2$ and 0.2% glucose (w/v). *Salmonella* Typhimurium SL3261 Δ*tolRA* Δ*msbB* [16] was grown in TYMC (10 g/liter tryptone, 5 g/liter yeast extract, 2 mM $MgCl_2$, and 2 mM $CaCl_2$) containing 0.2% glycerol (w/v) at 30°C. Antibiotics ampicillin (100 μg/ml), kanamycin (25 μg/ml) and chloramphenicol (30 μg/ml) were added to culture medium where appropriate.

### Reagents and antisera

Plasmid DNA Miniprep kit was obtained from Thermo Fisher Scientific (Waltham, MA, USA). In-Fusion HD plus kit for seamless DNA cloning was purchased from Takara Bio (Mountain View, CA, USA), restriction enzymes were from New England Biolabs (Ipswich, MA, USA) and Coomassie Blue G250 was from BioRad (Hercules, California, USA). Unless stated otherwise, other reagents and chemicals used in this study were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

Polyclonal rabbit antisera against *C. trachomatis* MOMP and HtrA were gifts from Frank Follmann (Statens Serum Institut, Copenhagen, Denmark) and Wilhelmina Huston (University of Technology Sydney, Australia), respectively.[22,32] Anti-rabbit peroxidase IgG antibody generated in goats was supplied by Rockland (Limerick, PA, USA).

### Plasmid construction

Primers used for plasmid construction (Table S1) and the amino acid sequences of recombinant proteins are listed in the supplementary file. A plasmid encoding *C. trachomatis* SvD MOMP-derived CTH522[22] with a C-terminal SpyTag002[34] (CTH522-SpT2) under T7 promoter control was created by PCR. The construct also included a FLAG epitope for detection purpose. Initially, two *E. coli* codon-optimized synthetic DNA fragments were generated by Geneart (Thermo Fisher Scientific, MA, USA), respectively encoding CTH522 and FLAG-SpT2. Next, the fragments were fused and amplified by an overlap extension PCR procedure in which the two fragments were mixed with primers 1 and 2. The PCR product was ligated into the *Nco*I/*Hind*III sites of pET28-SpT2-LivJ[35] using In-Fusion cloning to yield pET28-CTH522-FLAG-SpT2.

Plasmid pET28-SpT2-HtrA-H6, encoding a proteolytically inactive variant of HtrA with an N-terminal His6× (H6)-SpT2

sequence (SpT2-HtrA) under T7 promoter control, was created using a three-step overlap extension PCR. Initially, an *E. coli* optimized synthetic DNA fragment encoding *C. trachomatis* SvD HtrA was purchased from Geneart. To introduce the S247A-encoding mutation,[32] a first PCR fragment was generated using the synthetic HtrA sequence as a template and primers 3 and 4. A second PCR fragment was generated by using the synthetic HtrA sequence as a template and primers 5 and 6. In a third PCR step, fragments 1 and 2 were mixed and combined with primers 3 and 6. The resulting product was inserted into the *Kpn*I/*Hind*III sites of pET28-SpT2-LivJ[35] using In-Fusion cloning to yield pET28-SpT2-HtrA-H6.

Plasmid pIBA-CTH522-H6, encoding CTH522 with a C-terminal H6 tag (CTH522-H6) under *tet* promoter control, was created by PCR. First, an *E. coli* codon-optimized synthetic DNA fragment encoding CTH522-H6 was ordered from Geneart. Next, the sequence was amplified by PCR using primers 7 and 8. The PCR product was inserted into the *Xba*I-*Hind*III sites of pASK-IBA3 (Göttingen, Germany) using In-Fusion cloning to yield pIBA-CTH522-H6.

Plasmid pET22-pelB-HtrA-H6, encoding wild type (wt)-HtrA with an N-terminal *pelB* signal peptide and a C-terminal H6 sequence (HtrA-H6) under control of the T7 promoter, was kindly provided by Wilhelmina Huston (University of Technology Sydney, Australia).[32]

### Recombinant protein expression, purification and analysis

ClearColi (DE3) cells carrying plasmids encoding CTH522-SpT2 or SpT2-HtrA were induced by addition of 0.5 mM IPTG for protein expression. After 2 h induction, cells were harvested by centrifugation (6,000 × *g*, 10 min, 4°C) and resuspended in a lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 10 µg/ml lysozyme). After incubation at 37°C for 1 h, they were lysed using a Branson Sonifier 250 (Branson Ultrasonics, Connecticut, USA). Whole-cell lysates (WCL) were subjected to centrifugation (15,000 × *g*, 10 min, 4°C) to pellet the aggregates of CTH522-SpT2 and SpT2-HtrA. Contaminants (e.g. DNA, ribosomal RNA, phospholipids, LPS, host proteases) were removed using a washing regime as previously described for the production of protein bodies.[36] Briefly, the aggregates were washed with 1% Triton X-100 to remove excess membrane material, 1 M urea to break low-affinity protein interactions, 1 M of NaCl to break potential electrostatic protein interactions, and PBS to remove remaining urea or salt.

CTH522-SpT2 and SpT2-HtrA aggregates were dissolved in 8 M urea for 30 min at room temperature (RT). Ultracentrifugation (100,000 × *g*, 30 min, 4°C) was performed to remove remaining insoluble material. To get rid of urea and allow refolding, solubilized CTH522-SpT2 was extensively dialyzed against TG buffer (25 mM Tris-HCl pH 8.8, 10% glycerol) using Spectra/POR dialysis membrane (Thermo Fisher Scientific, MA, USA). Solubilized SpT2-HtrA, which carried a H6-tag, was first purified under denaturing conditions by affinity chromatography using Talon Superflow medium (GE Healthcare, Life Sciences, USA) as per manufacturer's instructions. Eluted SpT2-HtrA was overnight dialyzed against PβG buffer (PBS pH 7.4, 150 mM NaCl, 20 mM β-mercaptoethanol, 10% glycerol) containing β-mercaptoethanol to prevent protein reaggregation. Dialyzed CTH522-SpT2 and SpT2-HtrA were subjected to centrifugation (15,000 × *g*, 15 min, 4°C) to remove potential aggregates and then used for coupling to OMVs.

To isolate CTH522-H6, ClearColi (DE3) cells harboring pIBA-CTH522-H6 were induced with anhydrotetracycline (0.2 mg/l) for 2 h. CTH522-H6 aggregates were collected, processed and solubilized as described above. Similar to SpT2-HtrA, soluble CTH522-H6 was purified by affinity chromatography using Talon Superflow medium under denaturing conditions. The eluate was dialyzed overnight against the TG buffer at 4°C before being used in immunoassays.

As a positive control for proteolytic activity, wt-HtrA was produced as described,[32] with some modifications. Briefly, ClearColi (DE3) cells containing pET22-pelB-HtrA-H6 were grown at 30°C before protein expression was induced with 0.1 mM IPTG for 2 h. Cells were harvested by centrifugation (6,000 × *g*, 10 min, 4°C), and a cleared lysate containing soluble wt-HtrA was obtained through subcellular fractionation, as described previously.[37] wt-HtrA-H6 was purified by affinity chromatography using Talon Superflow medium. The eluate was dialyzed against the TG buffer (pH 7.4) overnight at 4°C.

Concentration of purified proteins was determined by comparison with a BSA standard on Coomassie-stained SDS-PAGE gels. Densitometric scanning on Coomassie-stained gels was performed with a Bio-Rad GS-800 calibrated densitometer and then processed with ImageJ (http://imagej.nih.gov/ij/). GE Healthcare's AI600 Imager was used to capture images of immunoblotting.

### Casein zymography assay

Casein zymography was performed as described previously.[38] Briefly, 1.11 µg of soluble SpT2-HtrA and wt-HtrA were separated on an SDS-PAGE gel containing 0.1% β-casein (Carl Roth, Germany). The gel was run in semi-native buffer (25 mM Tris-HCl, 250 mM glycine) at 15 mA while the gel-cassette was water-cooled. Renaturation of resolved proteins was then accomplished by immersing the gel in a 2.5% Triton X-100 solution at RT for 40 min. To permit staining, the gel was equilibrated in developing buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij35) at RT for 30 min before being reincubated at 37°C overnight. Protein bands with β-casein proteolytic activity were visualized through their transparency following staining of the gel with Coomassie.[39]

### Isolation and conjugation of Salmonella OMV platform to antigens

Isolation of HbpD-SpC-decorated OMVs from *Salmonella* cells was described previously.[21] In short, bacteria carrying the plasmid pEH3-HbpD-SpC were grown at 30°C and induced for expression of HbpD-SpC in the presence of 0.1 mM IPTG. After induction for 16 h, cultures were subjected to centrifugation (6,000 × *g*, 10 min, 4°C) to remove the cells. The spent medium was passed through a 0.45 µm-pore-size filter

(Millipore) and centrifuged (235,000 × *g*, 60 min, 4°C) to sediment the OMVs. The OMVs were resuspended in PBS containing 15% glycerol.

Purified SpT2-tagged antigens were incubated for 18 h at 4°C in 4-fold molar excess over HbpD-SpC, exposed at the OMV surface. After 5-fold dilution with PBS, the reaction mixtures were passed through a 0.45-µm filter to remove potential aggregates. Antigen-conjugated OMVs and control OMVs not incubated with antigen were sedimented by ultracentrifugation (208,000 × *g*, 75 min, 4°C) after incubation in high-salt washing buffer (PBS, 550 mM NaCl) to remove the contaminants.

### Mouse immunization and tissue collection

Six to eight-week-old female B6C3F1 mice[40] were purchased from Envigo (United Kingdom). Experiments were conducted under the 3 R principle and mice were provided with water and food *ad libitum*. All animal experiments were approved by Utrecht Univeristy Animal Ethics Committee (AVD1080020198224).

Mice were immunized three times (two-week intervals) intranasally with OMVs in a volume of 5 µl (2.5 µl per nostril). Doses of 12 µg of OMVs (total protein content) were used based on preliminary studies with pneumococcal OMV vaccine candidates showing strong generation of cellular and humoral responses without inducing adverse events (WSPJ and JL, unpublished data). Ten days after the last immunization all mice were sacrificed. Spleens and draining cervical lymph nodes (cLNs) were collected and processed to single-cell suspensions by passage through a 70 µm-filter. Erythrocytes were lysed with ACK buffer (0.15 M $NH_4Cl$, 1 mM $KHCO_3$, 0.1 mM $Na_2EDTA$, pH 7.3). Blood was drawn from the orbital plexus and collected in MiniCollect tubes (Greiner Bio-One), incubated at RT for 1 h and centrifuged at 12,000 × *g* at 4°C for 15 min to collect sera. Vaginal fluids were collected via washing the vaginal lumen twice with 50 µl ice-cold PBS with 1× protease inhibitor (Sigma-Aldrich), samples were cleared by centrifugation (10,000 × *g*) at 4°C for 10 min.

### Antigens specific enzyme linked immunosorbent assay (ELISA)

Antigen-specific antibodies in sera and vaginal washes were detected by ELISA.[22] Costar® assay plates (Corning) were coated with 1 µg/ml of purified CTH522 (Figure S5) or HtrA (Figure S2) in carbonate buffer pH 9.6, overnight at 4°C. Plates then were blocked with 200 µl/well dilution buffer (PBS, 0.5% Tween-20, 3% BSA-free protease) for 1.5 h at 37°C. Samples were added in duplicate in serial 2-fold dilutions in 100 µl/well and plates were incubated for 2 h at RT, followed by incubation for 1 h with HRP-conjugated anti-mouse IgG, IgG1 or IgG2a (BD Biosciences, dilution 1:2000) at RT. To assess the presence of secreted IgA in vagina, biotinylated anti-mouse IgA (BD Biosciences; diluted 1:2000) was added, followed by avid-HRP (BD Biosciences; diluted 1:1000). Plates were washed 5× with the dilution buffer and then developed with a 3,3′,5,5′-Tetramethylbenzidine solution for 30 min after which the reaction was blocked using $2NH_2SO_4$. The OD was recorded with iMark™ Microplate Absorbance Reader (Bio-Rad) at 450 nm (correction at 570/620 nm).

### Released cytokine quantification (Luminex)

To measure antigens specific cytokine production, cLN cells or splenocytes ($1 \times 10^6$) were incubated for 72 h with or without 10 µg/ml of purified CTH522, HtrA and OVA in RPMI media supplemented with GlutaMAX (Thermo Fisher Scientific), 10% FCS (Lonza) and penicillin/streptomycin. Collected cell culture supernatants were applied for cytokine analysis using the Magpix (Luminex XMAP) system.[41] In brief, the supernatants were incubated with magnetic beads coated with cytokine-specific antibodies, in polystyrene, black, 96-well flat bottom plates (Greiner Bio-One), overnight at 4°C. Biotin-conjugated detection antibodies and Streptavidin-PE (BD Biosciences) were used to detect the cytokines present on the beads. Antibody pairs used for IFN-γ, IL-17a, IL-4 and IL-5 cytokine detection are listed in the supplementary file (Table S2). Cytokine concentration was calculated using standard curves of the corresponding recombinant proteins, provided by the manufacturer. Median fluorescent intensity (MFI) data were analyzed using a 5-parameter logistic method (xPONENT software, Luminex, Austin, USA).

### Intracellular cytokine staining/flow cytometry (ICS/FC)

For intracellular cytokine staining, splenocytes ($1 \times 10^6$) were incubated with 10 µg/ml of purified CTH522, HtrA, OVA or 10 µg/ml of synthetic peptides (Table S3) in RPMI media containing 10 µM monensin (eBioscience) at 37°C for 6 h. Cells then were stained with ViaKrome808 (Beckman Coulter, Indianapolis, IN, USA), fluorescence-conjugated anti-mouse CD4 (clone RM 4–5; PerCP; BD Bioscience), and CD8 (clone Ly-2; FITC; eBioscience) antibodies in the presence of FcR-blocking anti-mouse CD16/32 antibody (clone 2.4G2; made in house) at 4°C for 30 min. Cells were fixed, permeabilized following manufacturer's instructions (BD Bioscience), and stained using anti-mouse IFN-γ (clone XMG1.2; PE; Invitrogen) at 4°C for 45 min. Samples were measured using a Beckman Coulter Cytoflex LX and data were analyzed by FlowJo Software v.10.7 (FlowJo LLC, Ashland, OR, USA). The percentage of intracellular cytokine-expressing $CD4^+$ or $CD8^+$ T-cell subsets in spleens was calculated by subtracting background of cells incubated without stimuli per individual mouse.[42]

### Statistical analysis

Data are shown as mean values plus standard error of mean (SEM). Statistical significance of differences between OMV-CTH522/OMV-HtrA and OMV-Ctrl vaccinated groups were determined using the analysis of variance (ANOVA) and Sidak's multiple comparisons test in GraphPad Prism version 8.0. Levels of statistical significance are denoted by asterisks: $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$ and $^{****}p < .0001$.

## Results

### Production of OMV-based *C.trachomatis* vaccine formulations

As a first step toward creating chlamydial antigen-decorated OMVs, MOMP-derived CTH522 and proteolytic inactive (S247A) HtrA were equipped with an N-terminal and C-terminal SpT2 ligation tag, respectively.[34] The resulting CTH522-SpT2 and SpT2-HtrA fusions (Figures S1A and S2A) were expressed in ClearColi(DE3), a host strain with genetically modified LPS that does not trigger endotoxic responses in human cells.[33] Analysis of CTH522-SpT2 expression using SDS-PAGE and Coomassie staining revealed that the 59 kDa fusion protein mostly accumulated in the pellet fraction after low-speed centrifugation of the whole-cell lysate, indicating extensive aggregation (Figure S1B, lane 3). This is consistent with previous reports on the production of the CTH522 subunit vaccine,[22] and we were unable to produce sufficient soluble material by altering growth and induction conditions. Therefore, we employed urea extraction to solubilize the aggregates and facilitate OMV coupling. After solubilization, the sample was dialyzed to remove urea and allow for the refolding of CTH522-SpT2 into a soluble conformation. Indeed, the protein was predominantly detected in the supernatant fraction following ultracentrifugation of the dialyzed sample (Figure S1C-D). Similarly, SpT2-HtrA formed aggregates upon expression (Figure S2B, lane 3), which may be attributed to the S247A substitution as previous studies have reported reduced solubility in *C. trachomatis* and *Helicobacter pylori* HtrA active site mutants.[32,38] Thus, we tried urea extraction to obtain the soluble antigen, similar to CTH522-SpT2. As SpT2-HtrA carried a H6-tag (Figure S2A), an additional affinity purification step was included (Figure S2C). Following dialysis, ~20% of soluble SpT2-HtrA could be recovered from the post-centrifugation supernatant (Figure S2C, lane 5; Figure S2D).

To confirm the proteolytic inactivity of purified SpT2-HtrA, we conducted an *in-gel* zymography assay.[43] *C. trachomatis* wild-type HtrA (wt-HtrA) was expressed in soluble form, purified (Figure S3)[32] and analyzed in parallel as a control (Figure S4). As expected, wt-HtrA showed strong proteolytic activity toward β-casein, resulting in clearance zones at the expected migration position (53 kDa) upon Coomassie staining (Figure S4, lane 1). In contrast, clearance zones were not observed for solubilized SpT2-HtrA (Figure S4, lane 3), confirming proteolytic inactivity of the mutant and suitability for use in a vaccine formulation.

OMV-based *C. trachomatis* vaccine formulations were generated using SpT2/SpC protein ligation technology.[21] Recombinant *Salmonella* OMVs displaying HbpD-SpC were incubated with purified CTH522-SpT2 or SpT2-HtrA (Figure 1(a,b)). Successful conjugation of the antigens to OMVs was visualized on Coomassie-stained SDS-PAGE gels as a band shift of the HbpD-SpC-SpT2-antigen adduct compared to the uncoupled HbpD-SpC protein, proportional to the molecular mass of the SpT2-antigen fusions (Figure 1(c,d), c.f. lanes 1 and 2). Conjugation appeared efficient as virtually no unconjugated HbpD-SpC was detected after incubation with the antigens. In both formulations, some excess unconjugated antigen was also detected, most likely corresponding to material that remained associated with the OMV surface despite high salt extraction of the OMVs after the coupling reaction (Figure 1(c,d), lane 2). In conclusion, two OMV-based chlamydial vaccine candidates were successfully produced for further immunogenicity analysis.

### Generation of specific systemic and mucosal antibodies

Humoral immune responses play an important role in reducing bacterial shedding and neutralizing *C. trachomatis* early infections.[22] Antibodies in systemic and local compartments, along with Th1 cell-mediated responses, are believed to contribute to the eradication of bacteria post-infection.[44,45] To examine the ability of OMV-based *C. trachomatis* vaccines to elicit humoral responses, female B6C3F1 mice were intranasally immunized with OMV-CTH522, OMV-HtrA, or control OMVs containing the HbpD carrier only (OMV-Ctrl) (Figure 2). Mice were sacrificed 10 d after the third immunization, and ELISA was performed to determine CTH522-specific and HtrA-specific total serum IgG titers. Importantly, no signs of adverse effects to the immunizations were observed for any of the OMV formulations.

Compared to the control group, mice vaccinated with OMV-HtrA exhibited significant levels of HtrA-specific serum IgG titers (Figure 3(c); $p < .0001$), while an increase in CTH522-specific serum IgG titers was observed in OMV-CTH522 vaccinated mice, although not statistically significant (Figure 3(a)). The systemic immune responses to the OMV vaccines were further characterized by analyzing IgG isotypes. Compared to OMV-Ctrl, the OMV-HtrA formulation induced significant levels of HtrA-specific serum IgG1 (Figure 3(g); $p < .0001$) and IgG2a (Figure 3(h); $p < .001$). The OMV-CTH522 vaccine showed an insignificant increase in serum IgG1 (Figure 3(e)) and IgG2a (Figure 3(f)) specific to CTH522. Notably, the levels of IgG2a specific to the antigens were higher than those of IgG1 at the same dilutions, indicating a primary Th1-driven antibody response.

To assess antibody responses at mucosal sites distal to the site of vaccination, in particular the genital surface, levels of vaginal IgA and IgG were measured 10 d after vaccination with the OMV formulations. The vaccine formulations elicited significant levels of vaginal IgG specific to either CTH522 (Figure 3(b), $p < .01$) or HtrA (Figure 3(d), $p < .0001$). However, vaginal IgA could not be clearly detected in any of the vaccinated mice (data not shown). Overall, intranasal administration of the OMV-CTH522 and OMV-HtrA formulations induced strong antigen-specific humoral responses in both the systemic and targeted vaginal compartments.

### Induction of specific cellular responses

The induction of specific cellular immunity, involving polyfunctional $CD4^+$ and $CD8^+$ T cells, is crucial for the clearance of *C. trachomatis* latent infection.[22,25,46] To investigate the cellular responses elicited by the OMV vaccines, cells derived from cLNs and spleens of vaccinated mice were stimulated with purified CTH522, HtrA, or OVA for 72 h. The levels of cytokines released into the cell culture supernatants were measured using Luminex.

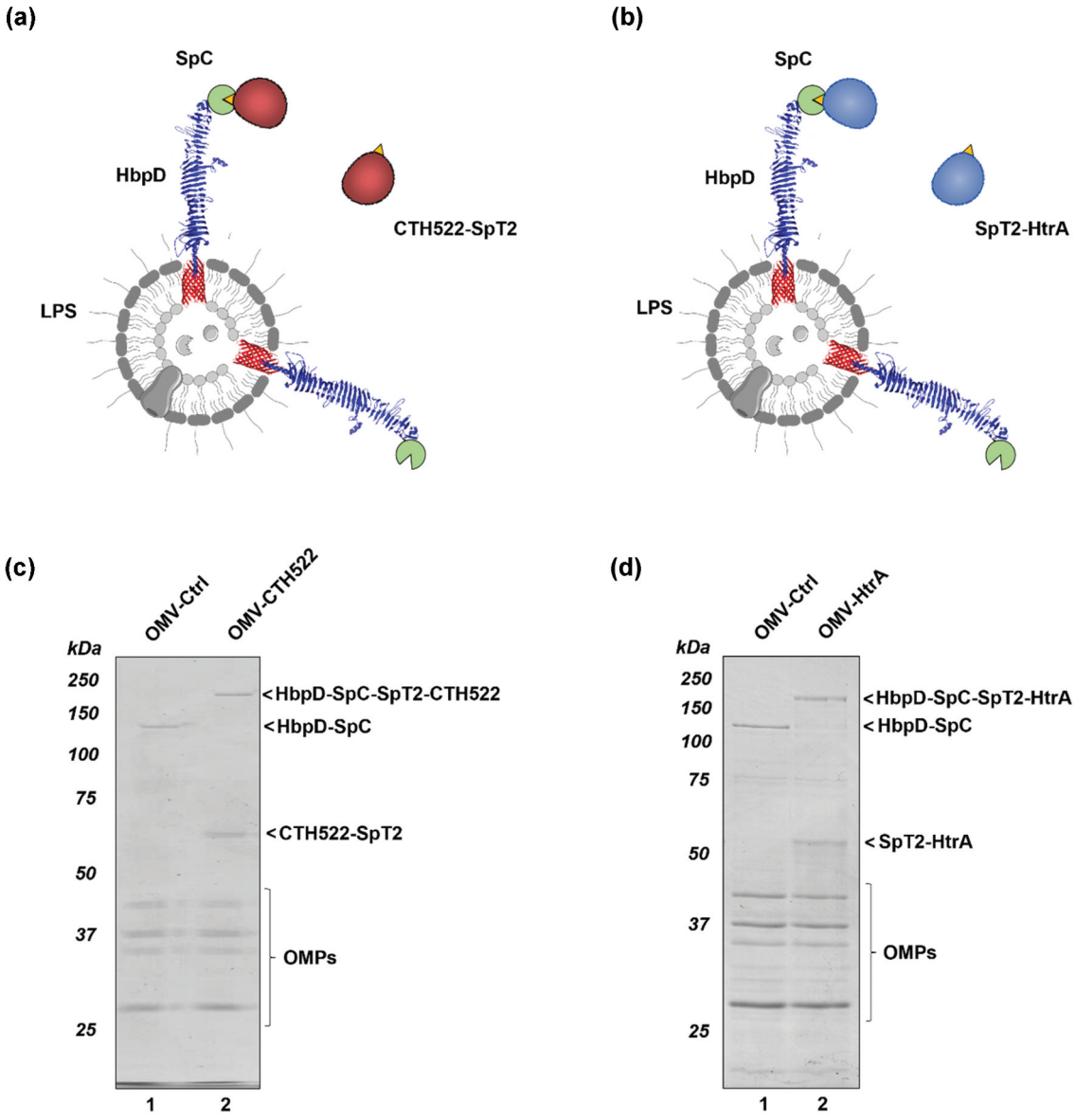


**Figure 1.** Production of OMV-based *C. trachomatis* vaccines. (a-b) Schematic representation of the strategy for vaccine production. Soluble CTH522-SpT2 (*A*) or SpT2-HtrA (B) are conjugated to *Salmonella* OMVs displaying HbpD-SpC to yield the OMV-CTH522 and OMV-HtrA vaccine formulations. (c-d) Protein profiles of OMV-CTH522 (*C*) and OMV-HtrA (*D*) compared to non-conjugated control OMVs (*OMV-Ctrl*) as analyzed by SDS-PAGE and Coomassie staining. Adducts (*HbpD-SpC-SpT2-CTH522*, *HbpD-SpC-SpT2-HtrA*), non-conjugated HbpD-SpC, non-conjugated antigens (*CTH522-SpT2*, *SpT2-HtrA*) and the major outer membrane proteins (*OMPs*) as present in the OMV membrane are indicated. Molecular weight markers (kDa) are indicated at the left side of the panels.

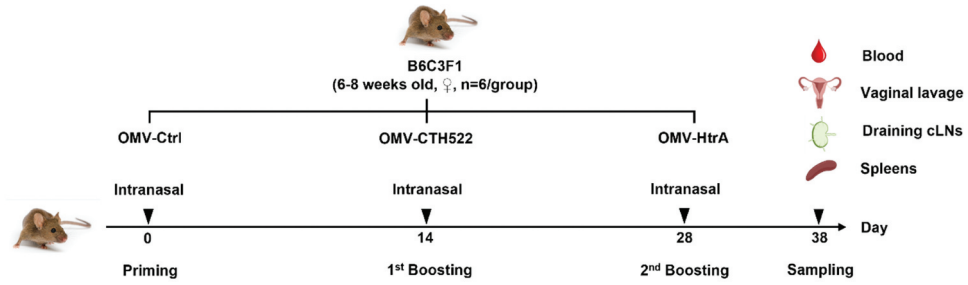


**Figure 2.** Intranasal immunization strategy and mouse tissue collection. B6C3F1 female mice were primed on day 0 and received two subsequent boosters on days 14 and 28, with each administration comprising 12 µg of either OMV-CTH522, OMV-HtrA, or OMV-Ctrl. Serum, vaginal fluid, draining cervical lymph nodes (cLns), and spleens of the vaccinated mice were collected 10 d after the third immunization.

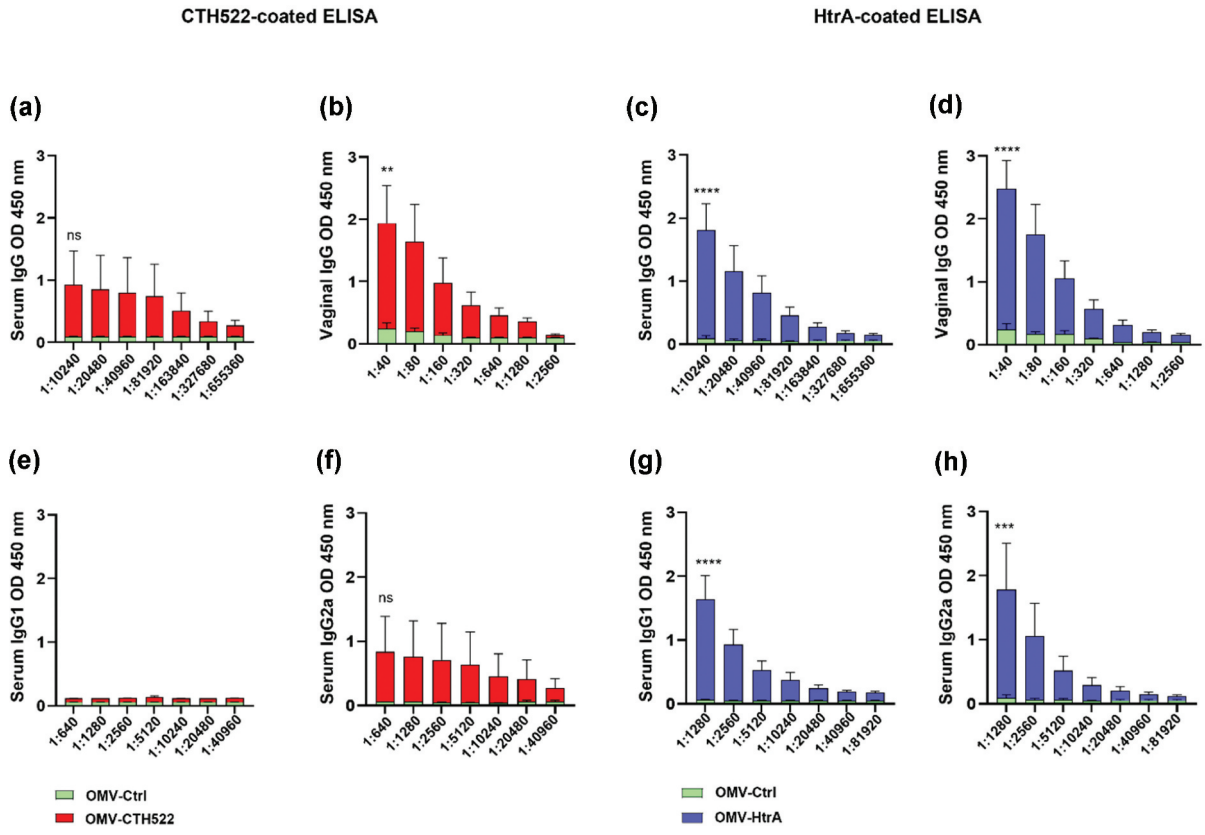


**Figure 3.** Humoral immune responses to *C. trachomatis* antigens. ELISA was used to assess antibody responses. (a-b) Post-vaccination kinetics of total serum and vaginal IgG against CTH522. (c-d) Post-vaccination total serum and vaginal IgG against HtrA. (e-f) pattern of IgG1 and IgG2a antibody responses specific to CTH522 in serum post-vaccination. (g-h) Post-vaccination IgG1 and IgG2a antibody responses specific to HtrA in serum. The error bars represent the standard error of mean (SEM). GraphPad Prism version 8.0 was used to analyze the statistically significant difference between the OMV-CTH522/OMV-HtrA and OMV-Ctrl vaccinated groups using ANOVA and Sidak's multiple comparisons test. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$ and $^{ns}$ (not significant).

Overall, there was a trend toward increased levels of secreted IFN-γ and IL-17a (Figure 4). Compared to the OMV-Ctrl group, cells from cLNs and spleens of mice vaccinated with OMV-CTH522 exhibited significantly higher levels of IL-17a (Figure 4(b); $p < .0001$ and 4(f); $p < .05$) upon stimulation with CTH522. Stimulated splenocyte cultures also showed significantly enhanced IFN-γ levels (Figure 4(e); $p < .01$), while a trend of IFN-γ increase was observed in cLN cell cultures (Figure 4(a)). In OMV-HtrA vaccinated mice, significant levels of HtrA-specific IL-17a (Figure 4(d); $p < .05$ and 4(h); $p < .001$) and IFN-γ (Figure 4(c); $p < .05$ and 4(g); $p < .0001$) were detected in both splenic and cLN cell cultures compared to the control samples. There was a slight increase in antigen-unspecific IFN-γ levels in splenocyte cultures of OMV-Ctrl vaccinated mice stimulated with CTH522 and HtrA (Figure 4(e,g)), possibly due to contaminants from the bacterial expression host that co-purified with the proteins used for restimulation (Figures S2 and S5). Negligible levels of secreted IFN-γ and IL-17a were detected in cLN and spleen cell cultures stimulated with non-related OVA control antigen, confirming the specificity of the cytokine responses. Specific IL-4 and IL-5 could not be detected in any of the samples (data not shown). Taken together, these results indicate that *Salmonella* OMV-based *C. trachomatis* vaccines induce robust Th17- and Th1-skewed responses, which likely contribute to the IgG2a subclass-switch (see Figure 3(f,h)).

To determine the specificity of OMV-primed IFN-γ/CD4$^+$ and IFN-γ/CD8$^+$ T cells, we used the NetMHC3.4/ANN prediction algorithm[47] to predict CTH522 and HtrA epitopes presented by the B6C3F1 mouse MHC (Table S3). Spleen cells from vaccinated mice (10 d post-vaccination; Figure 2) were restimulated for 6 h with purified CTH522, HtrA, OVA, or the predicted peptides and then analyzed using ICS/FC (Figure S6). In the OMV-CTH522 vaccinated mouse group, stimulation of splenocytes with either soluble CTH522 or MOMP$^{132-139}$ triggered slightly more IFN-γ production among the CD4$^+$ T cell population than stimulation with the irrelevant OVA peptide (Figure S6A-C). Additionally, there was a slight increase in percentage of CD8$^+$ splenocytes producing IFN-γ detectable upon restimulation with MOMP$^{132-139}$ compared to OVA (Figure S6D), indicating that OMV-CTH522 stimulates both IFN-γ expressing CD4$^+$ and CD8$^+$ T cell responses. These align with the predominant release of Th1-related IFN-γ above (Figure 4). The frequencies of HtrA-specific IFN-γ/CD4$^+$ and -CD8$^+$ T cells did not differ between OMV-HtrA and OMV-Ctrl vaccinated mice in this assay (Figure S7). In summary, the data demonstrate that both OMV vaccines induce IFN-γ/CD4$^+$ and IFN-γ/CD8$^+$ T cell responses, although OMV-CTH522 was more effective than OMV-HtrA.

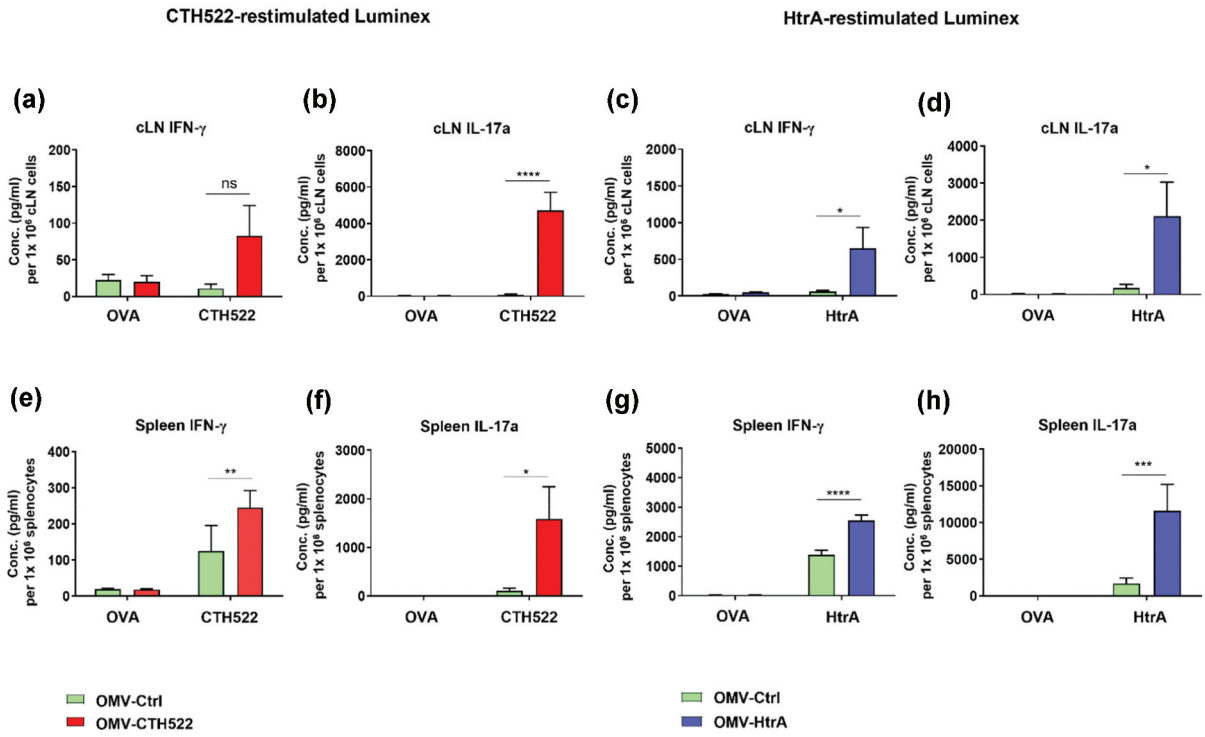


**Figure 4.** Antigen-specific cytokine released in the culture supernatants of cLN and splenic cells. Splenic and cLN cell suspensions ($1 \times 10^6$) were restimulated *in vitro* for 72 h with purified CTH522, HtrA, or OVA and released cytokines in cell culture supernatants were measured using Luminex assay. Levels of IFN-γ and IL-17a cytokines secreting from cLN cells (a-b) and splenocytes (e-f) after CTH522 and OVA stimulation. Levels of IFN-γ and IL-17a cytokines secreting from cLN cells (e-f) and splenocytes (g-h) after restimulation with HtrA and OVA. The standard error of mean (SEM) was used to express the results. ANOVA and Sidak's multiple comparisons tests were used in GraphPad prism version 8.0 to determine statistically significant differences between the OMV-CTH522/OMV-HtrA and OMV-Ctrl vaccinated groups. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$ and $^{ns}$ (not significant).

## Discussion

To address the pressing need for a *C. trachomatis* vaccine, we conducted a study to evaluate the design and immunogenicity of two recombinant OMV-based subunit vaccines using a mouse model. Intranasal administration of *Salmonella* OMVs displaying either CTH522 or HtrA antigens effectively stimulated specific humoral and cellular immune responses, thereby generating potential for future challenge studies. Native or recombinant OMVs are increasingly recognized as potential vaccines for mucosal applications due to their intrinsic adjuvant properties, compatibility with large-scale production, thermal stability, and importantly, their ability to enhance both systemic and local adaptive immune responses.[10–12] Compared to traditional parenteral vaccination regimes, intranasal administration is particularly attractive as it offers a needle-free approach, improving patient compliance, and takes advantage of the extensive and well-vascularized mucosal surface. Furthermore, the immune connection between mucosal sites allows intranasal administration to elicit immune responses in distant mucosal areas such as the colorectal and genital regions.[48] Given that *C. trachomatis* primarily initiates infection at the urogenital surface, the induction of local immunity through intranasal administration is highly advantageous for achieving effective and long-lasting protection against the pathogen invasion. This presents a clear benefit over parenteral vaccines, which typically elicit poor immunity at the mucosa.[49]

In a previous study, we successfully achieved high-level expression of wt-MOMP in an attenuated *Salmonella* strain.[37] However, when we transferred the construct to an isogenic hypervesiculating *Salmonella* vaccine strain, the overproduced wt-MOMP protein was not incorporated into OMVs (data not shown). Similarly, our attempts to express other *C. trachomatis* outer membrane protein antigens, such as wt-PmpD,[50] in OMVs were unsuccessful. We hypothesized that this failure may be due to a mismatch between the relatively long fatty acid chains of chlamydial lipid A[51] and the thinner hydrophobic core of the *Salmonella* outer membrane. Furthermore, we were unable to express HtrA in the lumen of OMVs upon fusion to an *E. coli* OmpA signal peptide[52] or at the surface of OMVs using the *Borrelia burgdorferi* OspA lipoprotein signal peptide,[53] which has been used to translocate other heterologous antigens to the *E. coli* OMV surface (data not shown).

To overcome challenges in expressing antigens in OMVs, we adopted a "plug-and-display" approach. This involved the separate expression and purification of the antigens, followed by their conjugation to the HbpD carrier displayed on the surface of OMVs using the SpT/SpC protein ligation system.[21] Instead of using wt-MOMP, we used a derivative construct called CTH522 with SpT2, which had been previously expressed as protein bodies, solubilized, and used in a clinical trial.[25] For HtrA, we expressed an active site mutant with SpT2. Despite the tendency of both *C. trachomatis* proteins to form aggregates during overexpression in *E. coli*, we

successfully purified soluble SpT2-tagged antigens through denaturation and refolding methods. Subsequently, these soluble antigens were conjugated to preexisting batches of the OMV-HbpD-SpC carrier. The coupling procedure demonstrated feasibility and robustness. A caveat of the procedure is that we cannot be certain that the antigens, though soluble, were refolded into a native-like conformation. Nevertheless, a similar protocol was used to express and purify recombinant CTH522, which successfully induced neutralizing antibodies in rodents and humans.[22,25]

Intranasal administration of our *C. trachomatis* vaccines resulted in specific immune responses at the local area, with particularly high IgG titers observed in the vaginal compartment, which are not generally achieved through systemic immunization.[54] Previous studies have also demonstrated the synergistic effect of combining systemic (prime) and mucosal (boost) vaccination to reach complete protective immunity against *C. trachomatis*, but this often requires complex injection and dosing strategies.[44–57] In our study, the IgG titers detected in the mouse vagina appeared to correlate with an increase in serum IgG titers. It is important to note that the adaptive immune system has limited regional specialization in the genito-urinary tissues.[58] Therefore, the increased levels of vaginal IgG antibodies specific to chlamydial antigens are likely derived from the circulation although, based on our data, the local production by resident plasma B cells cannot be excluded. Unlike nasal, lung, or intestinal fluids, semen and cervico-vaginal lavages typically contain lower levels of secretory IgA compared to IgG.[58] We consistently observed low levels of vaginal IgA titers specific to CTH522 and HtrA, which is in accordance with previous findings.[59] Although the role of vaginal IgA antibodies in protection is still a topic of debate, elevated IgG titers at the genital surface have proven to be important for neutralizing *C. trachomatis* and other sexually transmitted pathogens such as *Neisseria gonorrhoeae* and human immunodeficiency virus (HIV).[60]

Both the OMV-CTH522 and OMV-HtrA vaccines were found to promote biased responses of $CD4^+$ Th1 and Th17 cells. This was evidenced by significant levels of antigen-specific IFN-γ and IL-17a in the culture media of cells derived from draining cLNs and spleens. While both OMV vaccines stimulated splenic T cells to release IFN-γ, the presence of peptide-specific IFN-γ-expressing $CD4^+$ and $CD8^+$ T cells was only observed in the OMV-CTH522 vaccinated mouse groups. We hypothesize that the presence of CTH522-specific IFN-γ/$CD4^+$ T cells accounts for the detected IgG2a isotype switching in these mice. Additionally, the production of CTH522-specific IFN-γ/$CD8^+$ T cells may reflect the cross-presentation of OMV-associated antigens on MHC class I molecules, as previously observed.[17] The OMV-HtrA vaccine also induced detectable *ex vivo* HtrA-specific IFN-γ production, indicating $CD4^+$ Th1 cell responses.

Studies so far have not unanimously identified a specific immunogenicity profile correlating with protection to *C. trachomatis* infection. In humans, a humoral response, particularly IgA in cervical secretion, correlates with bacterial clearance.[61] In both human and mice a Th1/Th17 skewed $CD4^+$ T cell response seems essential for immune protection.[62–64] The role of $CD8^+$ T cells is less clear, although $CD8^+$ T cell responses played a pivotal role in a non-human primate model, i.e. CD8 depletion abrogated the protective effect of previous vaccination.[41,63] In this animal model, it was also shown that both cellular $CD4^+$ and $CD8^+$ T cell responses in combination with IgG1 antibody response accelerated the clearance of infection.[65] As described above, in our study, OMV-vectored vaccination was able to induce both a Th1/Th17-skewed immune response and an antigen-specific IgG immune response systemically and locally. This immune profile is in good agreement with the presumed correlates of protection and underscores the potential use of OMVs in *C. trachomatis* vaccine development.

Interestingly, the short 8-mer $MOMP^{132\text{-}139}$ peptide was able to stimulate both antigen-specific $CD4^+$ and $CD8^+$ T cell responses (see Figure S6). The apparent overlap of MHC-I and MHC-II epitopes seems counterintuitive, particularly because the latter are typically between 13 and 25 residues. Yet, the MHC-II binding core usually is much smaller,[66] possibly down until a length of five amino acids. For instance, searching the IEBD (settings: T cell & MHC binding, MHC II, mouse) with filter on 5-mer peptides returns three hits. When filtering for peptides with a length of eight amino acids, 847 records are found. In addition, antigens usually contain both $CD4^+$ and $CD8^+$ T cell epitopes, although the antigen processing pathways differ. As both pathways are by no means 100% efficient, it is conceivable that different molecules follow different routes. As an example, in an HPV16 mouse tumor model, the E7 oncoprotein was found to contain the sequence DRAHYNIVTF comprising a 7-mer helper T cell epitope (DRAHYNI) overlapping with a 9-mer CTL epitope (RAHYNIVTF).[67] Thus, although less common, $CD4^+$ T cell epitopes may be smaller than 9 amino acids and may overlap with $CD8^+$ T cell epitopes.

In conclusion, our proof-of-concept study provides evidence for the potential of the *Salmonella* OMV platform in the development of intranasal *C. trachomatis* vaccines. Due to their intrinsic adjuvant properties, OMVs displaying CTH522 elicited strong humoral and cellular immune responses, including both $CD4^+$ Th1/Th17 and $CD8^+$ T cells. On the other hand, OMV-HtrA primarily enhanced humoral responses and stimulated $CD4^+$ Th1/Th17 immune responses in mice. Whether these responses translate to actual protection against chlamydia, and how the candidate vaccines stand up against e.g. the existing adjuvanted CTH522 subunit vaccine,[22] remains to be tested by *in vivo* challenge studies. These are not trivial since vaginal infection with *C. trachomatis*, a human pathogen, does not lead to an ascending infection or tubal pathology in mice. On the other hand, rodent-specific counterpart *C. muridarium* is controlled by a different immune response, making this pathogen not representative for human *Chlamydia* infection.[64,68] To address these limitations, an intracervical murine infection model has been developed in which *C. trachomatis* infection in mice upper genital tract can be established.[62,69] We aim to use this model to evaluate the efficacy of the OMV vaccine formulations, both individually and in combination, including the exploration of a single, bivalent formulation incorporating both *C. trachomatis* antigens coupled to the same OMV.[70]

## Acknowledgments

We are grateful to Wilhelmina Houston for providing wt-HtrA constructs and advice. We thank Gregory M. Koningstein for performing the casein zymography assay, and Irene S. Ludwig and Danille ter Braake for their help with the mouse experiments. We also thank the Flow Cytometry and Cell Sorting Facility at Utrecht University for their assistance.

## Author contributions

Conceptualization, methodology, validation, formal analysis, investigation, data curation, D.T.H., E.N., L.M., P.V.U., W.S.P.J., A.J.A.M.S. and J.L.; writing – original draft preparation, D.T.H., E.N., W.S.P.J., A.J.A.M.S. and J.L.; writing – review and editing, D.T.H., E.N., P.V.U., W.S.P.J., A.J.A.M.S. and J.L.; visualization, supervision, project administration, funding acquisition, W.S.P.J., A.J.A.M.S. and J.L. All authors have read and agreed to the published version of the manuscript.

## Disclosure statement

J.L., W.S.P.J., and D.T.H. are involved in Abera Bioscience AB that aims to exploit the presented technology.

## Funding

D.T.H, E.N, and L.M received funding from the European Union's Horizon 2020 Research and Innovation Program via the Marie Skłodowska-Curie project VacPath [Grant agreement No. 812915].

## References

1. Newman L, Rowley J, Hoorn SV, Wijesooriya NS, Unemo M, Low N, Stevens G, Gottlieb S, Kiarie J, Temmerman M. Global estimates of the prevalence and incidence of four curable sexually transmitted infections in 2012 based on systematic review and global reporting. PLoS One. 2015;10:1–17. doi:10.1371/journal.pone.0143304.
2. Schautteet K, De Clercq E, Vanrompay D. Chlamydia trachomatis vaccine research through the years. Infect Dis Obstet Gynecol. 2011;2011:1–9. doi:10.1155/2011/963513.
3. Christensen S, McMahon RM, Martin JL, Huston WM. Life inside and out: making and breaking protein disulfide bonds in Chlamydia. Crit Rev Microbiol. 2019;45:33–50. doi:10.1080/1040841X.2018.1538933.
4. Panzetta ME, Valdivia RH, Saka HA. Chlamydia persistence: a survival strategy to evade antimicrobial effects *in-vitro* and *in-vivo*. Front Microbiol. 2018;9:1–11. doi:10.3389/fmicb.2018.03101.
5. Borges ÁH, Follmann F, Dietrich J. Chlamydia trachomatis vaccine development–a view on the current challenges and how to move forward. Expert Rev Vaccines. 2022;21(11):1555–1567. doi:10.1080/14760584.2022.2117694.
6. Murray SM, McKay PF. Chlamydia trachomatis: cell biology, immunology and vaccination. Vaccine. 2021;39(22):2965–2975. doi:10.1016/j.vaccine.2021.03.043.
7. Wong WF, Chambers JP, Gupta R, Arulanandam BP. Chlamydia and its many ways of escaping the host immune system. J Pathog. 2019;2019:1–9. doi:10.1155/2019/8604958.
8. Azegami T, Yuki Y, Kiyono H. Challenges in mucosal vaccines for the control of infectious diseases. Int Immunol. 2014;26(9):517–528. doi:10.1093/intimm/dxu063.
9. Brandtzaeg P. Induction of secretory immunity and memory at mucosal surfaces. Vaccine. 2007;25(30):5467–5484. doi:10.1016/j.vaccine.2006.12.001.
10. Raeven RHM, Rockx-Brouwer D, Kanojia G, van der Maas L, Bindels THE, ten Have R, van Riet E, Metz B, Kersten GFA. Intranasal immunization with outer membrane vesicle pertussis vaccine confers broad protection through mucosal IgA and Th17 responses. Sci Rep. 2020;10(1):1–11. doi:10.1038/s41598-020-63998-2.
11. van der Ley PA, Zariri A, van Riet E, Oosterhoff D, Kruiswijk CP. An intranasal OMV-based vaccine induces high mucosal and systemic protecting immunity against a SARS-CoV-2 infection. Front Immunol. 2021 Aug 25;12:457644. doi:10.3389/fimmu.2021.781280.
12. Jiang L, Driedonks TAP, Jong WSP, Dhakal S, Bart van den Berg van Saparoea H, Sitaras I, Zhou R, Caputo C, Littlefield K, Lowman M, et al. A bacterial extracellular vesicle-based intranasal vaccine against SARS-CoV-2 protects against disease and elicits neutralizing antibodies to wild-type and Delta variants. J Extracell Vesicles. 2022;11(3):11. doi:10.1002/jev2.12192.
13. Sartorio MG, Pardue EJ, Feldman MF, Haurat MF. Bacterial outer membrane vesicles: from discovery to applications. Annu Rev Microbiol. 2021;75(1):609–630. doi:10.1146/annurev-micro-052821-031444.
14. Long Q, Zheng P, Zheng X, Li W, Hua L, Yang Z, Huang W, Ma Y. Engineered bacterial membrane vesicles are promising carriers for vaccine design and tumor immunotherapy. Adv Drug Deliv Rev. 2022;186:114321. doi:10.1016/j.addr.2022.114321.
15. Daleke-Schermerhorn MH, Felix T, Soprova Z, ten Hagen-Jongman CM, Vikström D, Majlessi L, Beskers J, Follmann F, de Punder K, van der Wel NN, et al. Decoration of outer membrane vesicles with multiple antigens by using an autotransporter approach. Appl Environ Microbiol. 2014;80(18):5854–5865. doi:10.1128/AEM.01941-14.
16. Kuipers K, Jong WSP, van der Gaast-de Jongh CE, Houben D, van Opzeeland F, Simonetti E, van Selm S, de Groot R, Koenders MI, Azarian T, et al. Th17-mediated cross protection against pneumococcal carriage by vaccination with a variable antigen. Infect Immun. 2017;85(10):1–13. doi:10.1128/IAI.00281-17.
17. Schetters STT, Jong WSP, Horrevorts SK, Kruijssen LJW, Engels S, Stolk D, Daleke-Schermerhorn MH, Garcia-Vallejo J, Houben D, Unger WWJ, et al. Outer membrane vesicles engineered to express membrane-bound antigen program dendritic cells for cross-presentation to CD8+ T cells. Acta Biomater. 2019;91:248–257. doi:10.1016/j.actbio.2019.04.033.
18. Kuipers K, Daleke-Schermerhorn MH, Jong WSP, ten Hagen-Jongman CM, van Opzeeland F, Simonetti E, Luirink J, de Jonge MI. Salmonella outer membrane vesicles displaying high densities of pneumococcal antigen at the surface offer protection against colonization. Vaccine. 2015;33:2022–2029. doi:10.1016/j.vaccine.2015.03.010.
19. Hays MP, Houben D, Yang Y, Luirink J, Hardwidge PR. Immunization with Skp delivered on outer membrane vesicles protects mice against enterotoxigenic Escherichia coli challenge. Front Cell Infect Microbiol. 2018;8:1–5. doi:10.3389/fcimb.2018.00132.
20. Phan TH, Kuijl C, Huyn D, Jong WSP, Luirink J, Van Ulsen P. Overproducing the BAM complex improves secretion of difficult-to-secrete recombinant autotransporter chimeras. Microb Cell Fact. 2021;20(1):1–17. doi:10.1186/s12934-021-01668-2.
21. van den Berg van Saparoea HB, Houben D, de Jonge MI, Jong WSP, Luirink J. Display of recombinant proteins on bacterial outer membrane vesicles by using protein ligation. Appl Environ Microbiol. 2018;84:1–17. doi:10.1128/AEM.02567-17.
22. Olsen AW, Follmann F, Erneholm K, Rosenkrands I, Andersen P. 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. J Infect Dis. 2015;212(6):978–989. doi:10.1093/infdis/jiv137.
23. Tagawa Y, Ishikawa H, Yuasa N. Purification and partial characterization of the major outer membrane protein of Haemophilus somnus. Infect Immun. 1993;61(1):91–96. doi:10.1128/iai.61.1.91-96.1993.

24. Rodríguez-Marañón MJ, Bush RM, Peterson EM, Schirmer T, de la Maza LM. Prediction of the membrane-spanning β-strands of the major outer membrane protein of Chlamydia. Protein Sci. 2009;11:1854–1861. doi:10.1110/ps.3650102.
25. Abraham S, Juel HB, Bang P, Cheeseman HM, Dohn RB, Cole T, Kristiansen MP, Korsholm KS, Lewis D, Olsen AW, et al. Safety and immunogenicity of the chlamydia vaccine candidate CTH522 adjuvanted with CAF01 liposomes or aluminium hydroxide: a first-in-human, randomised, double-blind, placebo-controlled, phase 1 trial. Lancet Infect Dis. 2019;19(10):1091–1100. doi:10.1016/S1473-3099(19)30279-8.
26. Backert S, Bernegger S, Skórko-Glonek J, Wessler S. Extracellular HtrA serine proteases: an emerging new strategy in bacterial pathogenesis. Cell Microbiol. 2018;20(6):1–9. doi:10.1111/cmi.12845.
27. Skorko-Glonek J, Zurawa-Janicka D, Koper T, Jarzab M, Figaj D, Glaza P, Lipinska B. HtrA protease family as therapeutic targets. Curr Pharm Des. 2012;19(6):977–1009. doi:10.2174/138161281131906003.
28. Skorko-Glonek J, Figaj D, Zarzecka U, Przepiora T, Renke J, Lipinska B. The extracellular bacterial HtrA proteins as potential therapeutic targets and vaccine candidates. Curr Med Chem. 2017;24(20). doi:10.2174/0929867323666161223145825.
29. Wu X, Lei L, Gong S, Chen D, Flores R, Zhong G. The chlamydial periplasmic stress response serine protease cHtrA is secreted into host cell cytosol. BMC Microbiol. 2011;11(1):11. doi:10.1186/1471-2180-11-87.
30. Huston WM, Theodoropoulos C, Mathews SA, Timms P. Chlamydia trachomatis responds to heat shock, penicillin induced persistence, and IFN-gamma persistence by altering levels of the extracytoplasmic stress response protease HtrA. BMC Microbiol. 2008;8(1):1–16. doi:10.1186/1471-2180-8-190.
31. Rantsi T, Land JA, Joki-Korpela P, Ouburg S, Hokynar K, Paavonen J, Tiitinen A, Puolakkainen M. Predictive values of serum chlamydia trachomatis troa and htra igg antibodies as markers of persistent infection in the detection of pelvic adhesions and tubal occlusion. Microorganisms. 2019;7(10):1–10. doi:10.3390/microorganisms7100391.
32. Huston WM, Swedberg JE, Harris JM, Walsh TP, Mathews SA, Timms P. The temperature activated HtrA protease from pathogen Chlamydia trachomatis acts as both a chaperone and protease at 37 °C. FEBS Lett. 2007;581(18):3382–3386. doi:10.1016/j.febslet.2007.06.039.
33. Mamat U, Woodard RW, Wilke K, Souvignier C, Mead D, Steinmetz E, Terry K, Kovacich C, Zegers A, Knox C. Endotoxin-free protein production—ClearColi$^{TM}$ technology. Nat Methods. 2013;10:916–916. doi:10.1038/nmeth.f.367.
34. Keeble AH, Banerjee A, Ferla MP, Reddington SC, Anuar INAK, Howarth M. Evolving accelerated amidation by SpyTag/SpyCatcher to analyze membrane dynamics. Angew Chem - Int Ed. 2017;56:16521–16525. doi:10.1002/anie.201707623.
35. van Beek LF, Surmann K, van den Berg van Saparoea HB, Houben D, Jong WSP, Hentschker C, Ederveen THA, Mitsi E, Ferreira DM, van Opzeeland F, et al. Exploring metal availability in the natural niche of streptococcus pneumoniae to discover potential vaccine antigens. Virulence. 2020;11:1310–1328. doi:10.1080/21505594.2020.1825908.
36. Van Beek LF, Langereis JD, Van Saparoea HBVDB, Gillard J, Jong WSP, Van Opzeeland FJ, Mesman R, Van Niftrik L, Joosten I, Diavatopoulos DA, et al. Intranasal vaccination with protein bodies elicit strong protection against Streptococcus pneumoniae colonization Intranasal vaccination with protein bodies elicit strong protection against Streptococcus pneumoniae colonization. Vaccine. 2021;39:6920–6929. doi:10.1016/j.vaccine.2021.10.006.
37. Huynh DT, Jong WSP, Koningstein GM, Van Ulsen P, Luirink J. Overexpression of the bam complex improves the production of Chlamydia trachomatis MOMP in the E. coli outer membrane. Int J Mol Sci. 2022;23(13):1–16. doi:10.3390/ijms23137393.
38. Zarzecka U, Modrak-Wójcik A, Figaj D, Apanowicz M, Lesner A, Bzowska A, Lipinska B, Zawilak-Pawlik A, Backert S, Skorko-Glonek J. Properties of the HtrA protease from bacterium Helicobacter pylori whose activity is indispensable for growth under stress conditions. Front Microbiol. 2019;10:1–16. doi:10.3389/fmicb.2019.00961.
39. Albrecht N, Tegtmeyer N, Sticht H, Skórko-Glonek J, Backert S. Amino-terminal processing of Helicobacter pylori serine protease HtrA: role in oligomerization and activity regulation. Front Microbiol. 2018;9:1–16. doi:10.3389/fmicb.2018.00642.
40. Badamchi-Zadeh A, McKay PF, Korber BT, Barinaga G, Walters AA, Nunes A, Gomes JP, Follmann F, Tregoning JS, Shattock RJ. A multi-component prime-boost vaccination regimen with a consensus MOMP antigen enhances Chlamydia trachomatis clearance. Front Immunol. 2016;7. doi:10.3389/fimmu.2016.00162.
41. Olivares-Zavaleta N, Whitmire WM, Kari L, Sturdevant GL, Caldwell HD. CD8 + T cells define an unexpected role in live-attenuated vaccine protective immunity against Chlamydia trachomatis infection in Macaques. J Immunol. 2014;192(10):4648–4654. doi:10.4049/jimmunol.1400120.
42. Platteel ACM, Marit de Groot A, Keller C, Andersen P, Ovaa H, Kloetzel PM, Mishto M, Sijts AJAM. Strategies to enhance immunogenicity of cDNA vaccine encoded antigens by modulation of antigen processing. Vaccine. 2016;34(42):5132–5140. doi:10.1016/j.vaccine.2016.08.039.
43. Harrer A, Boehm M, Backert S, Tegtmeyer N. Overexpression of serine protease HtrA enhances disruption of adherens junctions, paracellular transmigration and type IV secretion of CagA by Helicobacter pylori. Gut Pathog. 2017;9(1):1–12. doi:10.1186/s13099-017-0189-6.
44. Wern JE, Sorensen MR, Olsen AW, Andersen P, Follmann F. Simultaneous subcutaneous and intranasal administration of a CAF01-adjuvanted Chlamydia vaccine elicits elevated IgA and protective Th1/Th17 responses in the genital tract. Front Immunol. 2017;8:1–11. doi:10.3389/fimmu.2017.00569.
45. Dieu N, Tran N, Olsen AW, Follmann F. Th1/Th17 T cell tissue-resident immunity increases protection, but is not required in a vaccine strategy against genital infection with Chlamydia trachomatis. Front Immunol. 2021;12:1–12. doi:10.3389/fimmu.2021.790463.
46. Coleman MA, Lorenzen E, Contreras V, Olsen AW, Andersen P, Desjardins D, Rosenkrands I, Juel HB, Delache B, Langlois S, et al. Chlamydia trachomatis vaccination regimes induce antibody and T cell responses and accelerate clearance of infection in a non-human primate model. Front Immunol. 2022;13:1–13. doi:10.3389/fimmu.2022.1057375.
47. Lundegaard C, Lamberth K, Harndahl M, Buus S, Lund O, Nielsen M. NetMHC-3.0: accurate web accessible predictions of human, mouse and monkey MHC class I affinities for peptides of length 8-11. Nucleic Acids Res. 2008;36:509–512. doi:10.1093/nar/gkn202.
48. Du G, Qin M, Sun X. Recent progress in application of nanovaccines for enhancing mucosal immune responses. Acta Pharm Sin B. 2022;13(6):2334–2345. doi:10.1016/j.apsb.2022.08.010.
49. Knisely JM, Buyon LE, Mandt R, Farkas R, Balasingam S, Bok K, Buchholz UJ, Souza MPD, Gordon JL, King DFL, et al. Mucosal vaccines for SARS-CoV-2: scienti fi c gaps and opportunities — workshop report. NPJ Vaccines. 2022;8(1):1–7. doi:10.1038/s41541-023-00654-6.
50. Huynh DT, Jong WSP, Oudejans MAH, van den Berg van Saparoea HB, Luirink J, Van Ulsen P. Heterologous display of Chlamydia trachomatis PmpD passenger at the surface of Salmonella OMVs. Membranes. 2023;13(4):1–18. doi:10.3390/membranes13040366.
51. Ma H, Cummins DD, Edelstein NB, Gomez J, Khan A, Llewellyn MD, Picudella T, Willsey SR, Nangia S. Modeling diversity in structures of bacterial outer membrane lipids. J Chem Theory Comput. 2017;13(2):811–824. doi:10.1021/acs.jctc.6b00856.

52. Bartolini E, Ianni E, Frigimelica E, Petracca R, Galli G, Scorza FB, Norais N, Laera D, Giusti F, Pierleoni A, et al. Recombinant outer membrane vesicles carrying Chlamydia muridarum HtrA induce antibodies that neutralize chlamydial infection in vitro. J Extracell Vesicles. 2013;2. doi:10.3402/jev.v2i0.20181.
53. Irene C, Fantappiè L, Caproni E, Zerbini F, Anesi A, Tomasi M, Zanella I, Stupia S, Prete S, Valensin S, et al. Bacterial outer membrane vesicles engineered with lipidated antigens as a platform for Staphylococcus aureus vaccine. Proc Natl Acad Sci USA. 2019;116(43):21780–21788. doi:10.1073/pnas.1905112116.
54. Neutra MR, Kozlowski PA. Mucosal vaccines: the promise and the challenge. Nat Rev Immunol. 2006;6(2):148–158. doi:10.1038/nri1777.
55. Lorenzen E, Follmann F, Bøje S, Erneholm K, Olsen AW, Agerholm JS, Jungersen G, Andersen P. Intramuscular priming and intranasal boosting induce strong genital immunity through secretory IgA in minipigs infected with Chlamydia trachomatis. Front Immunol. 2015;6:1–12. doi:10.3389/fimmu.2015.00628.
56. Garcia-Del Rio L, Diaz-Rodriguez P, Pedersen GK, Christensen D, Landin M. Sublingual boosting with a novel mucoadhesive thermogelling hydrogel following parenteral CAF01 priming as a strategy against Chlamydia trachomatis. Adv Healthc Mater. 2022;2102508:1–9. doi:10.1002/adhm.202102508.
57. Kuczkowska K, Myrbråten I, Øverland L, Eijsink VGH, Follmann F, Mathiesen G, Dietrich J, Dean D. Lactobacillus plantarum producing a Chlamydia trachomatis antigen induces a specific IgA response after mucosal booster immunization. PLoS One. 2017;12(5):1–16. doi:10.1371/journal.pone.0176401.
58. Brandtzaeg P, Farstad IN, Haraldsen G. Regional specialization in the mucosal immune system: primed cells do not always home along the same track. Immunol Today. 1999;20(6):267–277. doi:10.1016/S0167-5699(99)01468-1.
59. Rose F, Wern JE, Gavins F, Andersen P, Follmann F, Foged C. A strong adjuvant based on glycol-chitosan-coated lipid-polymer hybrid nanoparticles potentiates mucosal immune responses against the recombinant Chlamydia trachomatis fusion antigen CTH522. J Controlled Release. 2018;271:88–97. doi:10.1016/j.jconrel.2017.12.003.
60. Mestecky J, Moldoveanu Z, Russell MW. Immunologic uniqueness of the genital tract: challenge for vaccine development. Am J Reprod Immunol. 2005;53(5):208–214. doi:10.1111/j.1600-0897.2005.00267.x.
61. Brunham RC, Kuo CC, Cles L, Holmes KK. Correlation of host immune response with quantitative recovery of chlamydia trachomatis from the human endocervix. Infect Immun. 1983;39(3):1491–1494. doi:10.1128/iai.39.3.1491-1494.1983.
62. Nguyen NDNT, Olsen AW, Lorenzen E, Andersen P, Hvid M, Follmann F, Dietrich J. Parenteral vaccination protects against transcervical infection with Chlamydia trachomatis and generate tissue-resident T cells post-challenge. NPJ Vaccines [Internet]. 2020;5(1):1–12. doi:10.1038/s41541-020-0157-x.
63. Liang S, Bulir D, Kaushic C, Mahony J. Considerations for the rational design of a Chlamydia vaccine. Hum Vaccin Immunother [Internet]. 2017;13:831–835. doi:10.1080/21645515.2016.1252886.
64. Brunham RC. Problems with understanding Chlamydia trachomatis immunology. J Infect Dis. 2022;225:2043–2049. doi:10.1093/infdis/jiab610.
65. Lorenzen E, Contreras V, Olsen AW, Andersen P, Desjardins D, Rosenkrands I, Juel HB, Delache B, Langlois S, Delaugerre C, et al. Multi-component prime-boost Chlamydia trachomatis vaccination regimes induce antibody and T cell responses and accelerate clearance of infection in a non-human primate model. Front Immunol. 2022;13. doi:10.3389/fimmu.2022.1057375.
66. Wang P, Sidney J, Dow C, Mothé B, Sette A, Peters B, Stormo G. A systematic assessment of MHC class II peptide binding predictions and evaluation of a consensus approach. PLoS Comput Biol. 2008;4(4):4. doi:10.1371/journal.pcbi.1000048.
67. Tindle RW, Croft S, Herd K, Malcolm K, Geczy AF, Stewart T, Fernando GJP, Hospital A. A vaccine conjugate of 'ISCAR' immunocarrier and peptide epitopes of the E7 cervical cancer-associated protein of human papillomavirus type 16 elicits specific Th1- and Th2-type responses in immunized mice in the absence of oil-based adjuvants. Clin Exp Immunol. 1995;101:265–271. doi:10.1111/j.1365-2249.1995.tb08349.x.
68. Sturdevant GL, Caldwell HD. Innate immunity is sufficient for the clearance of Chlamydia trachomatis from the female mouse genital tract. Pathog Dis. 2014;72(1):70–73. doi:10.1111/2049-632X.12164.
69. Rajeeve K, Sivadasan R. Transcervical mouse infections with Chlamydia trachomatis and determination of bacterial burden. Bio Protoc. 2020;10:10. doi:10.21769/BioProtoc.3506.
70. van den Berg van Saparoea HB, Houben D, Kuijl C, Luirink J, Jong WSP. Combining protein ligation systems to expand the functionality of semi-synthetic outer membrane vesicle nanoparticles. Front Microbiol. 2020;11:1–10. doi:10.3389/fmicb.2020.00890.