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Lack of neutralization of *Chlamydia trachomatis* infection by high avidity monoclonal antibodies to surface-exposed major outer membrane protein variable domain IV

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ABSTRACT

Chlamydia trachomatis is the leading cause of sexually transmitted diseases causing frequent, long-lasting, and often asymptomatic recurrent infections resulting in severe reproductive complications. *C. trachomatis* is an intracellular Gram-negative bacterium with a biphasic developmental cycle in which extracellular, infectious elementary bodies (EB) alternate with the intracellular replicating reticulate bodies (RB). The outer membrane of EB consists of a tight disulfide cross-linking protein network. The most essential protein is the 42 kDa major outer membrane protein (MOMP) that contributes to the rigid structural integrity of the outer membrane. MOMP is a transmembrane protein with a β-barrel structure consisting of four variable domains (VD) separated by five constant domains. VDIV possesses surface-exposed species-specific epitopes recognized by the immune system and, therefore, functions as a candidate for vaccine development. To analyze the protective contribution of antibodies for a MOMP vaccine, we investigated the specificity and binding characteristics of two monoclonal antibodies (MAb)224.2 and MAb244.4 directed against *C. trachomatis* serovar D MOMP. By immunoelectron microscopy, we found that both MAb bind to the surface of *C. trachomatis* EB. By epitope mapping, we characterized the MOMP epitope as linear consisting of 6 amino acids: ^{322}TIAGAGD^{328}. By ELISA it was shown that both antibodies bind with a higher avidity to the chlamydial surface compared to binding to monomeric MOMP, indicating that the antibodies bind divalently to the surface of *C. trachomatis* EB. Despite strong binding to the chlamydial surface, the antibodies only partially reduced the infectivity. This may be explained by the observation that even though both MAb covered the EB surface, antibodies could not be regularly detected on EB after the uptake into the host cell.

1. Introduction

Chlamydia trachomatis is causing the most significant bacterial sexually transmitted disease (STD) worldwide (Vasilevsky et al., 2014). *Chlamydia* are obligate intracellular Gram-negative bacteria with a complex developmental cycle, with two morphologically and functionally different forms: the extracellular non-replicating and infectious elementary bodies (EB), and the intracellular replicating reticulate bodies (RB). During the biphasic cycle, EB attach to receptive host cells and induce its uptake. Upon contact, the EB gain entry to the host cell using a vesicle transport system (S Birkelund et al., 1994; Murray and McKay, 2021). It causes asymptomatic infection in 75–90% of all cases of STD (Murray and McKay, 2021). Most women with urogenital *C. trachomatis* experience subclinical infections, and when left untreated it may result in chronic pelvic inflammatory disease eventually causing tubal scarring. Over time, scaring can result in long-term reproductive problems such as ectopic pregnancy and tubal factor infertility.

C. trachomatis species are grouped into three different biovars based on different infection-related disease phenotypes. These biovars are further divided into serovars based on the variable domains (VD) of *C. trachomatis* major outer membrane protein (MOMP) (Murray and McKay, 2021; Wang and Grayston, 1974).

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The outer membrane of *Chlamydia* EB can be purified using detergents, and this fraction is known as the chlamydial outer membrane complex (COMC), the dominant protein of which is MOMP, that accounts for 60% of the protein content (Caldwell et al., 1981). The rigid structure of the outer membrane results from a tight cross-linking network of disulfide bonds between its proteins providing both physical and chemical protection (Caldwell et al., 1981; Findlay et al., 2005; Hatch et al., 1986). The outer part of the membrane consists of lipopolysaccharide (LPS) closely associated to trimeric MOMP. A second family of proteins located in COMC is the nine polymorphic membrane proteins (Pmp) of approximately 100 kDa (Grimwood and Stephens, 1999). These proteins are auto-transporters that form a C-terminal β-barrel through the chlamydial outer membrane with a surface-exposed N-terminal parallel β-helix, named the passenger domain. The most cross-species conserved Pmp is PmpD. Coupling a fragment of PmpD passenger domain to micro beads, (Paes et al., 2018) showed that this resulted in adherence of the beads to host cells. In a mouse model, recombinant PmpD elicited protection against intra-vaginal challenge of *C. trachomaits* (Paes et al., 2016), showing that PmpD is crucial for attachment of *C. trachomatis* to host cells. In addition, the outer membrane contains a highly conserved type III secretion system (T3SS) with a needle-shaped complex capable of penetrating the plasma- or inclusion membrane to secrete effector proteins into the host cell (Hobolt-Pedersen et al., 2009; Nans et al., 2014; Vasilevsky et al., 2016). For uptake, *C. trachomatis* injects the actin-recruiting phosphoprotein (Tarp) into the host cell cytoplasm that induces uptake by actine polymerization, but other mechanisms as clathrin coated pits and pinocytosis are also active (Gitsels et al., 2019).

The COMC components OmcA and OmcB are crucial cysteine-rich proteins of 9 and 62 kDa, respectively. OmcB is extensively cross-linked in the periplasm of EB forming disulfide bonds with both MOMP and OmcA (Everett et al., 1994), and is therefore important for the structural integrity of EB.

MOMP functions as a porin and has several characteristics in common with the porins in the outer membrane of Gram-negative bacteria (Wyllie et al., 1998). This was confirmed by Findlay et al. (2005) who observed the conductance of ion flow through recombinant MOMP (rMOMP) reconstituted in planar lipid bilayers. It is thought to facilitate the transport of amino acids, peptides, and disaccharides over the RB membrane (Jones et al., 2000).

MOMP is the serotype defining antigen of *C. trachomatis* (Stephens et al., 1982). It has a trimeric structure with 16 antiparallel transmembrane β-sheets (Findlay et al., 2005) of which four are surface-exposed VD named I-IV, separated by five highly conserved constant domains. VDIV is the longest with 31 amino acids, and it possesses species-specific epitopes. All four surface-exposed loops vary between serovars and are highly immunogenic. The sequence variability of the VD is proposed to help *C. trachomatis* escape immunological surveillance (Findlay et al., 2005; Stephens et al., 1988, 1987; Sun et al., 2007). As MOMP can function as an antigen to generate serovar-specific antibodies, its epitopes are candidates for *C. trachomatis* vaccine development (Olsen et al., 2021).

In this study we investigated two monoclonal antibodies (MAb) against a surface exposed MOMP VDIV epitope and examined their binding affinity to *C. trachomatis*. The antibodies were generated by immunizing mice with purified *C. trachomatis* serovar D EB, which elicited the production of antibodies against the chlamydial surface. To selectively obtain antibodies to MOMP, the mouse was boosted with rMOMP so that only B-memory cells against linear MOMP epitopes were activated (Bumbueviciute et al., 2010). Hereby MAb224.2 and MAb244.4 were obtained and used for epitope mapping, binding characteristics, and their influence on *C. trachomatis* infection.

2. Materials and methods

2.1. Bacteria strains and cells

C. trachomatis D/UW-3/CX, E/Bour, L2/434/Bu, McCoy, HeLa, HaK and Ag8-NS1 were obtained from the American Type Culture Collection (ATCC, VA, USA).

2.2. Cultivation of Chlamydia trachomatis

For purification of RB or EB, McCoy cell monolayers were seeded in 6-well plates with a density of 5×10^4 cells/ml in RPMI 1640 with HEPES buffer and 5% FCS (infection medium) for 24 h at 37 °C and 5% CO_2. Semi-confluent monolayers were infected with 0.5 inclusion forming units (IFU) per cell. *C. trachomatis* serovar D and E diluted in infection medium and PBS (1 +1), were added to the plate, and centrifuged at 1000 x g for 30 min at 37 °C followed by a 30 min incubation at 37 °C and 5% CO_2. *C. trachomatis* serovar L2 was incubated for 60 min at 37 °C and 5% CO_2 without centrifugation. Medium was changed to infection medium with 1 μg/ml cycloheximide and addition of 0.1% glucose for 48 h (L2) or 68 h (D, E) at 37 °C and 5% CO_2. Purification of EB and RB was done according to Lausen et al. (2021), and the purity was controlled by negative staining and transmission electron microscopy.

2.3. Immunofluorescence staining and confocal microscopy

McCoy or HeLa cell monolayers on coverslips infected with *C. trachomatis* serovar D, E, or L2 were fixed in 3.7% formaldehyde in PBS for 20 min at 4 °C. The cells were permeabilized with 0.2% Triton X-100 in PBS for 7 min and blocked with 0.1% bovine serum albumin (BSA) for 15 min at 37 °C. Primary antibodies: MAb32.2 against MOMP (Allan C. Shaw et al., 2002a), MAb224.2, MAb244.4, or rabbit PAb17 against *C. trachomatis* L2 COMC (S Birkelund et al., 1994a) were diluted in 0.1% BSA in phosphate-buffered saline (PBS) and the samples were incubated for 30 min at 37 °C. The cells were washed three times in PBS and incubated with secondary antibodies: Goat anti-mouse IgG FITC-conjugated (#115–096–146), Goat anti-rabbit IgG TRITC-conjugated (#111–025–144), or Goat anti-rabbit IgG Alexa Fluor 647-conjugated (#111–605–144), (Jackson ImmunoResearch, Cambridge, UK) diluted 1:200 in PBS with 0.1% BSA for 30 min at 37 °C. For actin stanning, 1 μg/ml rhodamine phalloidin was added to the secondary antibodies. The cells were washed, and DNA was stained with 1 μg/ml To-Pro-3 iodide (ThermoFisher Scientific) or 4′,6-diamidino-2-phenylindole (DAPI)(Sigma-Aldrich) diluted in PBS for 10 min at room temperature. Coverslips were mounted on slides and the cells were imaged using a SP5 confocal laser scanning microscope (Leica microsystems, Welzlar, Germany) with HC PL APO 100x/1.47 oil immersion objective or Zeiss LSM 900 with 63x/1.40 oil immersion objective (Zeiss, Jena, Germany).

2.4. Immunoelectron microscopy

For immunoelectron microscopy (IEM) 5 μl of purified EB were mounted on a 400-mesh carbon-coated, glow-discharged nickel grids (SPI Supplies, PA) for 2 min. Grids were washed on three drops PBS and then blocked on one drop of 0.5% ovalbumin (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 2 min. Subsequently, the grids were incubated with primary antibody MAb32.2, MAb224.2, MAb244.4, MAb 15.1 or PAb17 (S. Birkelund et al., 1994b)(1:20 in ovalbumin buffer) for 30 min at 37 °C. This was followed by incubation with secondary goat anti-mouse/goat anti-rabbit antibody conjugated with 10 nm colloidal gold 1:25, (British BioCell, UK) for 30 min at 37 °C. The grids were washed on three drops PBS, then incubated on 3 drops 1% cold fish gelatin (Sigma-Aldrich) in PBS for 10 min each, washed on three drops PBS, stained with 0.5% or 1% (w/v) phosphotungstic acid at pH 7.0 and

blotted dry. Images were obtained with a transmission electron microscopy (JEOL 1400, Tokyo, Japan) operated at 60 keV and equipped with a TVIPS TemCam FX416 digital camera (TVIPS, Gauting, Germany), (Lausen et al., 2021).

2.5. Recombinant C. trachomatis D MOMP

The *C. trachomatis* serovar D *momp* gene was PCR amplified corresponding to amino acid 23–393 using primers that were extended with ligation-independent cloning sites. The PCR product was purified and cloned into pET30-Ek/LIC (Merck) which encodes an N-terminal histidine tag. The plasmid was purified, and control sequenced. For expression of rMOMP *E. coli* BL21/DE3 was used. The Histidine-tagged MOMP fusion protein was purified by affinity chromatography under denaturing conditions using Hi-Trap column (Cytiva) (Mygind et al., 1998).

2.6. Monoclonal antibodies

At week 9, 10, 11 after birth BALB/c mice were immunized intramuscularly with 30 μg of purified EB in PBS mixed 1 + 1 with Incomplete Freund's Adjuvant (Sigma Aldrich). At week 20, day 1 and 2, 30 μg of rMOMP in PBS were given intraperitoneally. At day 4 spleen cells were fused using polyethylene glycol 6000 with azaguanine resistant myeloma cell line, Ag8-NS1, and distributed on 96 well plates with murine peritoneal macrophages as previous described (Birkelund et al., 1988) For selection, aminopterin was used in RPMI 1640 with 10% fetal calf serum, hypoxanthine, thymidine, and 2 μg/ml gentamicin. Hybridomas were screened for antibody production using ELISA with wells coated with 1 μg/ml rMOMP, and horseradish peroxidase conjugated affinity purified goat anti-mouse IgG Fcγ fragment specific secondary antibody (Jackson ImmunoResearch). Subcloning was performed for antibody producing clones. Cultivation of antibodies was done in medium with gentamicin to avoid interference of penicillin in later *Chlamydia* experiments. For Biacore3000TM analysis, the MAb were purified from culture media using Recombinant Protein G - Sepharose™ 4B chromatography according to the manufacturer (ThermoFischer, USA).

2.7. Peptide synthesis

Peptides were synthesized using solid phase Fmoc chemistry on ABI model 433 A Peptide Synthesizer (Applied Biosystems – ThermoFisher Scientific, Waltham, MA). Peptides were purified on C18 column using high-performance liquid chronography (Waters, Wilmslow, United Kingdom). Peptide synthesis and purification were controlled by mass spectrometry using MALDI-TOF (Bruker Daltonics, Germany).

2.8. Biacore3000™ analysis

Real-time surface plasmon resonance was carried out using Biacore3000™ (Cytiva, Marlborough, MA) equipment (Axelsen et al., 2009). MAb224.2 and MAb244.4 were purified from culture medium using HiTrap® Protein G HP Columns according to the manufacturer's instructions (Merck, Kenilworth, NJ). Briefly, 20 μg/ml of each MAb was covalently immobilized in separate flow cells in a CM5 dextran sensor chip. Antibodies were diluted in a 10 mM sodium acetate buffer and the remaining carboxymethyl groups were blocked with 1 M ethanolamine. Antibody-peptide binding was analyzed at 25 °C and flow rate 5 μl/min Peptides were diluted in comprising 10 mM HEPES + 150 mM NaCl + 1 mM EGTA + 1.5 mM $CaCl_2$ + 0.005% Tween-20. Bound peptides were liberated with glycine-HCl pH 3.0. Data were evaluated using the BIAevaluation software from the manufacturer (Cytiva).

2.9. Competition ELISA with recombinant MOMP

Nunc™ MaxiSorp™ ELISA plates (ThermoFisher Scientific, Waltham, MA) were coated with 1 μg/ml rMOMP D in PBS at 4 °C overnight. Excess binding was blocked by adding 1% BSA in PBS to each well for 1 h at 37 °C. The plates were washed four times with PBS pH 7.2 with 0.05% Tween-20. Washing procedures were performed between each step. Synthetic peptides were diluted in PBS with 0.1% BSA and 0.05% Tween-20 to a concentration of 50 μM. They were added to the ELISA plate in a 2-fold-dilution, respectively, whereafter MAb224.2 or MAb244.4 were added to the plate diluted 1:400 in 0.1% BSA. The plates were incubated for 1 h at 37 °C. Then, the secondary goat anti-mouse IgG antibody HRP-conjugated (#115–035–062, Jackson ImmunoResearch Laboratories Inc., Cambridge, UK) was added diluted 1:30.000 in PBS with 0.1% BSA and 0.05% Tween-20, and incubated for 1 h at 37 °C. The enzymatic reaction was initiated by adding the HRP-substrate TMB-ONE (Kem-En-Tec, Tåstrup, Denmark) for 30 min at 37 °C. Subsequently, the reaction was stopped by adding 100 μl 1 M HCL. The plates were read on an Sunrise™ plate reader (Tecan, Männedorf, Switzerland) at 450 nm.

2.10. Competition ELISA with Chlamydia trachomatis EB

Competition ELISA with *C. trachomatis* EB was performed similarly to competition ELISA with rMOMP. One μg/ml purified *C. trachomatis* D EB was used as antigen. MAb224.2 or MAb244.4 were tested with a 4-fold-dilution series of peptides.

2.11. Neutralization assay

The HaK cell line was used because it does not express Fc gamma receptor that can interfere with the neutralization assay. Semi-confluent cell monolayers were seeded at a density of 5×10^4 cells/ml on coverslips. MAb were diluted 1/10 and incubated with increasing two-fold dilutions of purified *C. trachomatis* serovar D EB for 30 min at 37 °C before application to semi-confluent HaK cell monolayers (Su et al., 1991). After 48 h, immunofluorescence microscopy was used to detect *Chlamydia* inclusions. The percentage of infectivity inhibition was calculated: Inhibition % = [(Inclusions of control – Inclusions with MAb)/(Inclusions of control MAb)] * 100. As control, MAb35.5 specific for cytoplasmic chlamydial DnaK was used (S. Birkelund et al., 1994).

2.12. Chlamydia trachomatis D and L2 entry into HeLa or HaK cells in the presence of MAb224.2 and MAb244.4

C. trachomatis L2 and D, 10 CFU/cell, was mixed with 1/10 monoclonal antibody hybridoma supernatant of MAb224.2, MAb244.4, MAb15.1 or MAb35.2, and incubated for 30 min at 37 °C. The *C. trachomatis* D and L2: antibody mixtures were added to HeLa/HaK cell monolayers on coverslips and incubated for 2 hrs at 37 °C. The cells were washed 2 times in PBS and fixed in 3.7% formaldehyde in PBS for 20 min at 4 °C. Immunofluorescences staining was performed with PAb17, and to detect the binding of MAb and PAb, double staining with cross absorbed Goat anti mouse IgG FITC-conjugated (#115–096–146) and Goat anti-rabbit IgG Alexa Fluor 647-conjugated (#111–605–144) (Jackson ImmunoResearch) were used. Cell actin was visualized with phalloidin-rhodamine staining and cell nucleus with DAPI. Confocal microcopy was done using Zeiss and Leica confocal microscopes.

3. Results

3.1. Characterization and binding of monoclonal antibodies

After subcloning, 17 MAb against rMOMP were obtained, two reacted in ELISA with a peptide corresponding to VDI (aa 86–106), and 12 with a VDIV peptide (aa 307–338). Three MAb did not react with the used peptides. Due to their strong reactivity with *C. trachomatis* D, E and L2, MAb224.2 and MAb244.4 were selected for further experiments.

To characterize binding of MAb224.2 (Fig. 1A, D, C) and MAb244.4 (Fig. 1 B, E, H) to MOMP, high-resolution confocal immunofluorescence

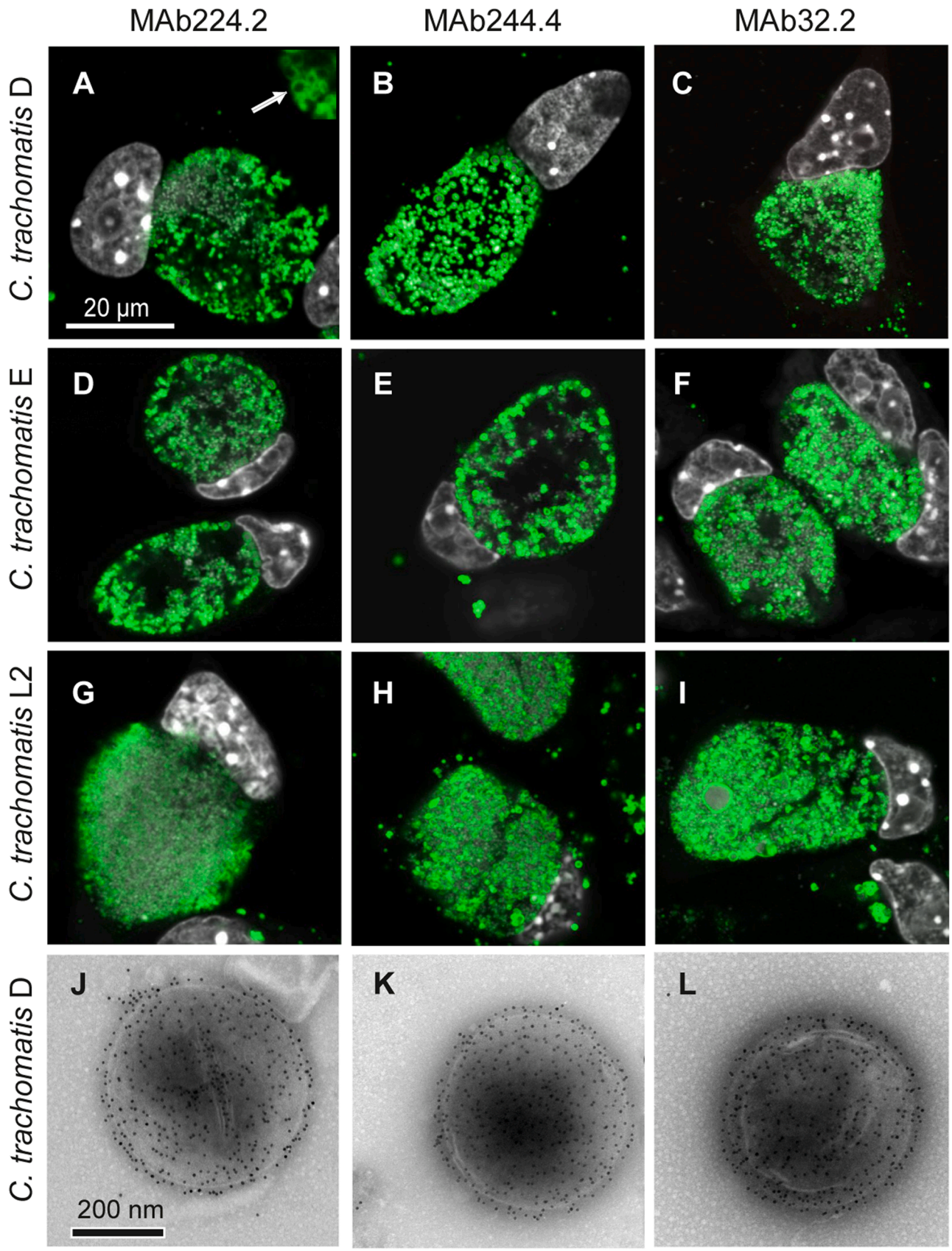


Fig. 1. Antibody reaction to *C. trachomatis*. Immunofluorescence microscopy of McCoy cell monolayers infected with *C. trachomatis* D, E, or L2 for 48 hrs. stained with primary antibodies: MAb224.2 (A, D, G), MAb244.4 (B, E, H), or MAb32.2 (C, F, I) (green), DNA stained To-Pro-3 (white), bar 20 µm. Insert, shows immunostaining chlamydial membrane arrow (A). Immunoelectron microscopy of purified *C. trachomatis* D EB reacted with MAb224.2 (J), MAb244.4 (K), or MAb32.3 (L) and visualized by 10 nm immune gold secondary antibody, bar 200 nm.

microscopy with *C. trachomatis* D, E, or L2 infected cells (48 h) was performed. As a positive control MAb32.2 against MOMP was used (Fig. 1C, F, I), (Allan C Shaw et al., 2002b). In agreement with MOMP localization in the outer membrane MAb224.2 and MAb244.4 reacted with the circumference of individual RB or EB of *C. trachomatis* D, E, and L2 (Fig. 1A, insert), like the reaction with MAb32.2.

By immunofluorescence microscopy, the cells were fixed and permeabilized. To determine whether antibodies reacted with the native chlamydial surface IEM was performed with unfixed purified EB. Purified EB reacted with MAb224.2 and MAb244.4 like the reaction with MAb32.2 specific for MOMP as immunogold particles covered the complete surface of the EB (Fig. 1J-L) demonstrating that the MOMP epitopes are surface exposed.

3.2. Limitation of antibody epitopes on Chlamydia trachomatis serovar D MOMP

To limit of the epitopes for MAb224.2 and MAb244.4, they were tested by ELISA with 4 peptides of *C. trachomatis* serovar D MOMP VDIV, all ending at amino acid 337, but shortened from the N-terminal part (Fig. 2, line 1–4). The shortest peptide the antibodies reacted with was from amino acid 321–337 (Fig. 2, line 3). At amino acid 331 and 332 *C. trachomatis* E and L2 sequences differ from serovar D (Fig. 2 line 5–6). Both MAb224.2 and MAb244.4 reacted with serovar D, E, and L2 (Fig. 1). Therefore, these amino acids are unlikely part of the epitope (Fig. 2). Hereby, the epitopes for MAb224.2 and MAb244.4 could be limited to the region ^{321}PTIAGAGDVK^{330} of VDIV MOMP.

3.3. Analysis of monoclonal antibody binding affinity

Real-time surface plasmon resonance was used to examine the binding kinetics and affinity between the purified MAb224.2 or MAb244.4 and MOMP. The antibodies were used as ligands on the sensor and the synthetic peptides of *C. trachomatis* D VDIV (amino acid 307–337) as analyte. The analysis was performed and the interaction between immobilized MAb and the soluble peptides of VDIV was measured by injection of peptides at different concentrations. To obtain sensograms, the *C. trachomatis* D MOMP VDIV peptide was applied, and association and dissociation rates were measured (Fig. 3).

The sensograms from Fig. 3A and B show real-time binding kinetic measurements between the MAbs and the peptide corresponding to MOMP VDIV. Sensogram association rates (k_{on}) and resonance units were nearly identical for the two antibodies. The dissociation rates (k_{off}) for the antibodies matched the association rates. The injection was stopped before full equilibrium was obtained, but the dissociation constant (K_D) could be calculated (Fig. 3C). Both MAb224.2 and MAb244.4 had low K_D (20.2 and 9.6 nM, respectively), implying that the antibodies had good binding affinity (Fig. 3).

1	MOMP D	^{307}PKSATAIFDTTTLNPTIAGAGDVKTGAEGQL^{337}	+
2	MOMP D	^{317}TTLNPTIAGAGDVKTGAEGQL^{337}	+
3	MOMP D	^{321}PTIAGAGDVKTGAEGQL^{337}	+
4	MOMP D	^{325}GAGDVKTGAEGQL^{337}	-
5	MOMP E	^{307}PKSATAIFDTTTLNPTIAGAGDVKASAEGQL^{337}	+
6	MOMP L2	^{307}PKSATTVFDVTTLNPTIAGAGDVKASAEGQL^{337}	+
7	MOMP epitope	^{321}**PTIAGAGDVK**^{330}	

Fig. 2. Epitope limitation. 1–4: Decreasing length of synthetic peptides covering amino acid 307–337 of *C. trachomatis* MOMP VDIV. The reaction with MAb224.2 and 244.4 is marked with + /-.
5–6: alignment of *C. trachomatis* serovar E and L2 VDIV, amino acids are compared to *C. trachomatis* D. Changes are indicated in red. 7: In bold is shown the region of the antibody's epitopes.

3.4. Epitope mapping by Alanine-scan and competition ELISA

To map the contribution to binding of the individual amino acids in VDIV, competition ELISA was performed. Using the mapped epitope region to be between ^{321}PTIAGAGDVK^{330} of *C. trachomatis* serovar D MOMP, 15′mer peptides were synthesized with one amino acid exchanged in each position of 316–330 to either A or G (Alanine-scan, Table 1) using a starting concentration of 50 μM peptide and 2-fold-dilutions. The synthetic peptides in suspension were mixed with the antibodies and the inhibitory effect of each peptide was measured on ELISA plates coated with rMOMP. For each peptide concentration the measured OD was plotted on graphs (Fig. 4). The graphs in Fig. 4A (MAb224.2) and B (MAb244.4) show the competitive inhibition by the antibodies. The 15′mer unmodified ^{316}TTTLNPTIAGAGDVK^{330} peptide demonstrates full competition of the binding to rMOMP at 25 μM. When ^{325}G was changed to A, no inhibition was observed indicating that ^{325}G is a crucial amino acid for binding of the antibodies. This was also the case for ^{323}I changed to A. Change of ^{324}A and ^{327}G also showed marked effect and change of ^{326}A and ^{322}T showed reduced binding. Similar results were obtained with MAb244.4 (Fig. 4B). Therefore, the epitope was mapped to ^{322}TIAGAGD^{328} for both antibodies.

3.5. Determination of monoclonal antibody binding avidity to the Chlamydial surface

To compare binding of the antibodies to monomeric rMOMP and to the surface of EB, ELISA plates were coated with rMOMP and EB, respectively, and thereafter competition ELISA was performed using MAb224.2 and MAb244.4 and the unmodified peptide ^{316}TTTLNPTIAGAGDVK^{330}. Thereby a possible divalent binding to the EB surface (binding avidity) could be addressed.

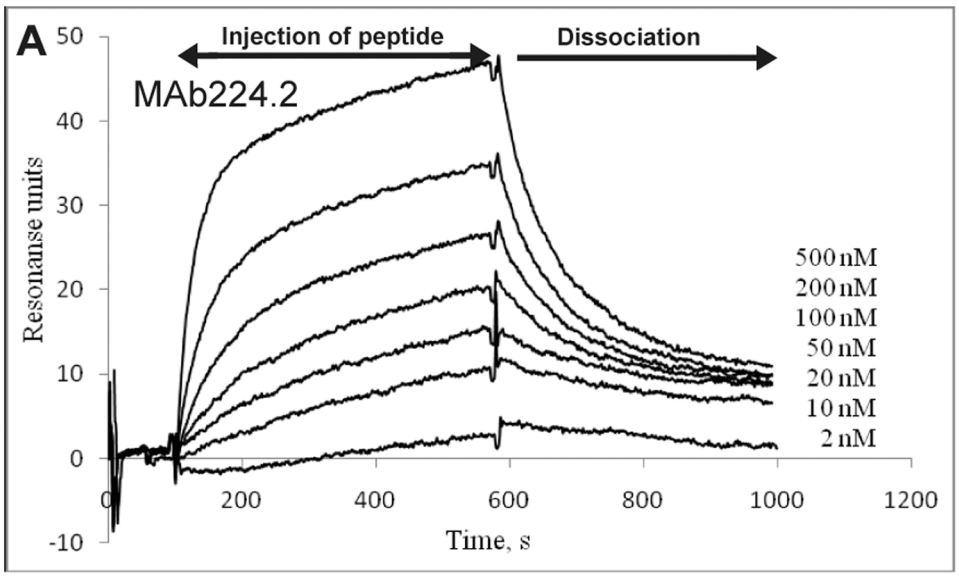
Fig. 5 shows the binding of MAb224.2 and MAb244.4 to either *C. trachomatis* D rMOMP or to EB. The competition curves for rMOMP and EB are shifted right for MAb224.2, showing a change in binding to EB in which a high density of MOMP is present on its surface. Thus, divalent binding contributes to the binding (Fig. 5, blue lines). For MAb244.4 a major right shift was observed between the two curves, where up to 32 times more peptide was necessary to obtain the same inhibition (Fig. 5, green lines). This indicates that both MAb244.4 antigen binding sites can bind to two MOMP molecules on the chlamydial surface simultaneously, resulting in high avidity. Even though the epitopes for the two antibodies were identical, the subclasses differed. MAb224.2 was IgG2a and MAb244.4 was IgG1. This could be due to different flexibility in the hinge region and thereby account for the difference in avidity.

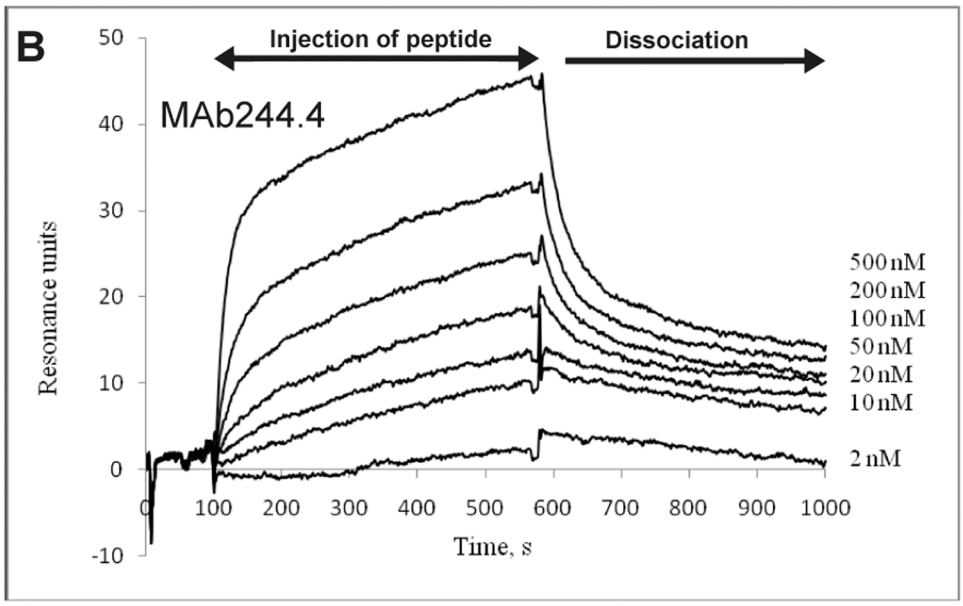
3.6. Neutralization of Chlamydia trachomatis by MAb224.2 and MAb244.4

To investigate whether the antibodies directed against MOMP VDIV had a neutralizing effect on the infection, a neutralization assay with MAb224.2 and MAb244.4 was performed. Each MAb was incubated with *C. trachomatis* D and added to semi-confluent HaK cell monolayers for 48 h, HaK cells lack Fc-receptors, whereby Fc mediated uptake cannot influence the neutralization assay (Su et al., 1991). MAb35.2 against the cytoplasmic *C. trachomatis* L2 DnaK protein was used as control (Birkelund et al., 1990). The number of inclusions was determined by immunofluorescence microscopy. The results show an inhibition of inclusion forming units to 38% for both MOMP VDIV antibodies compared to the control. Even though MAb244.4 had a high avidity, both antibodies showed similar low reduction in infectivity.

3.7. Uptake in HeLa cells of Chlamydia trachomatis L2 and D treated with MAb224.2 and MAb244.4

To analyze for a possible mechanism used by chlamydial EB to





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	k_{on}, $M^{-1}s^{-1}$	k_{off}, s^{-1}	K_D, nM
MAb224.2	$1.25x10^{5}$	$2.53x10^{-3}$	20.2
MAb244.4	$1.37x10^{5}$	$1.31x10^{-3}$	9.6

Fig. 3. Biacore3000™ analysis**.** Real-time binding kinetics between MAb224.2 (A) and MAb244.4 (B) and the synthetic peptide MOMP VDIV amino acid 307–337. The concentration of peptide added is indicated on the right of the sensogram. Association and dissociation rates were measured for each concentration. (C) Calculated results from sensograms of association rate k_{on} dissociation rate k_{off} and dissociation constant K_D.

escape antibody neutralization, we investigated chlamydial entry into HeLa cells in the presence of the MAb224.2, MAb244.4 or MAb 35.2 against DnaK. The respective antibodies were incubated with *C. trachomatis* L2 and D EB and added to a monolayers of HeLa cells. After incubation, the cells were washed and fixed with formaldehyde, and thus, bound monoclonal antibodies were fixed to both extracellular and intracellular *C. trachomatis* EB. Hereafter, the samples were stained using PAb17 (rabbit antibody to *C. trachomatis* L2 COMC). By use of secondary goat anti-mouse and goat anti-rabbit antibodies, all chlamydiae were visualized by the COMC antibody (red), and the MAb binding was identified by the FITC-conjugated anti-mouse antibody (green) (Fig. 6).

Fig. 6 shows MAb224.2 (A) and MAb244.4 (B) binding to EB. Green stained *C. trachomatis* L2 EB are located on the cellular membrane, while red stained EB are located closer to the nucleus. Some double-stained chlamydiae (yellow) are also seen (Fig. 6A-B). Most of the intracellular EB were stained red, indicating loss of MAb on the intracellular EB, while EB attached to the surface of HeLa cells were green. Thus, when

Table 1
Peptides used for epitope mapping.

AA Exchange	Sequence
AA 316–330	TTTLNPTIAGAGDVK
^{321}P ⇒ A	TTTLN**A**TIAGAGDVK
^{322}T ⇒ A	TTTLNP**A**IAGAGDVK
^{323}I ⇒ A	TTTLNPT**A**AGAGDVK
^{324}A ⇒ G	TTTLNPTI**G**GAGDVK
^{325}G ⇒ A	TTTLNPTIA**A**AGDVK
^{326}A ⇒ G	TTTLNPTIAG**G**GDVK
^{327}G ⇒ A	TTTLNPTIAGA**A**DVK
^{328}D ⇒ A	TTTLNPTIAGAG**A**VK
^{329}V ⇒ A	TTTLNPTIAGAGD**A**K

Synthetic peptides corresponding to C. trachomatis serovar D MOMP VDIV. The 15′mer peptide 316TTTLNPTIAGAGDVK330 had one amino acid changed at each position of 321–329 to either A or G depending on the primary sequence.

C. trachomatis L2 EB covered by MAb224.2 or MAb244.4 are entering HeLa cells, the MAb can no longer attach to the EB surface. *C. trachomatis* L2 infection can be obtained without centrifugation in contrary to the trachoma and genital serovars. Infection with serovar D using centrifugation resulted in similar result for both antibodies (Fig. 6C, D). The control with MAb35.2 against DnaK, also used for the neutralization assay, showed no reaction with EB, only reaction with PAb17 was seen, in agreement with the cytoplasmic localization of DnaK (Fig. 6E), control without MAb (Fig. 6F), shows red stained EB and the control without L2 EB show no staining (Fig. 6G). Similar results were obtained for *C. trachomatis* D controls (Data not shown).

To eliminate the possibility that the antibodies did not bind to the smallest EB, immunoelectron microscopy was performed also with *C. trachomatis* L2, which shows that the surface of all EB were covered with MAb224.2, MAb244.4, and PAb17 (Fig. 6 H-J). Thereby, it could be excluded that the missing reaction to intracellular EB was due to lake of binding of the antibodies to EB.

To investigate whether antibodies to other surface antigens exhibited a similar phenomenon as the MOMP antibodies, MAb15.1 to LPS was used. LPS in *C. trachomatis* is rough with lipide-A and core oligosaccharide but without an O-chain (Nurminen et al., 1985), and the core-oligosaccharide has a genus specific epitope (Brade et al., 1987). MAb15.1 specific for *Chlamydia* LPS and reacts with the surface of *C. trachomatis* L2 EB, but LPS is detached when EB are incubated with MAb15.1 (Birkelund et al., 1989), and this was also the case for *C. trachomatis* D (Fig. 7A). The detachment of LPS from the EB surface resulted in a reaction that was not as clear as for the MOMP antibodies, but similar results were obtained (Fig. 7B), indicating the same mechanism for both MOMP and LPS antibodies.

3.8. Uptake in HaK cells of Chlamydia trachomatis L2 and D treated with MAb224.2 and MAb244.4

HeLa cells express Fcγ receptor III (FcγRIII) (Su et al., 1991), and

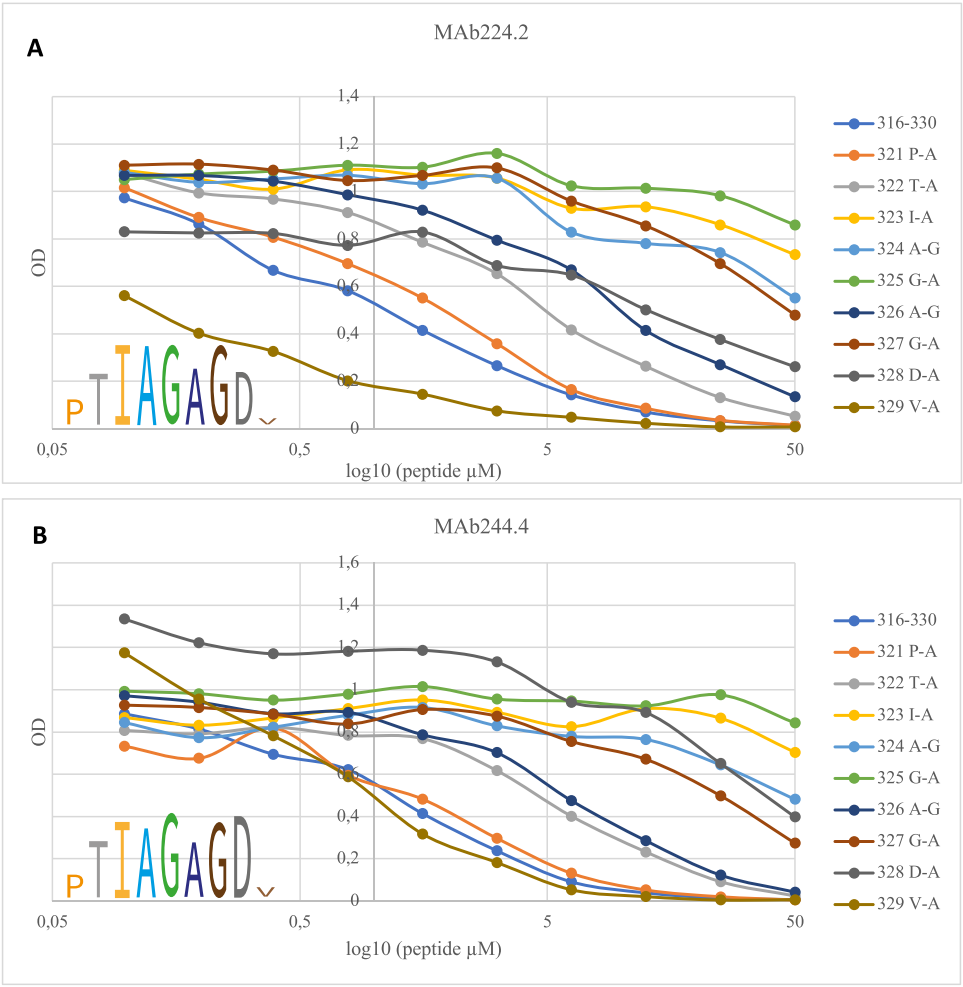

Fig. 4. Competition ELISA. ELISA trays were coated with rMOMP and the inhibitory effect of peptides (Table 1) in concentration of 0.1–50 µM were measured for the binding of MAb224.2 (A) or MAb244.4 (B) to rMOMP. The unchanged peptide (blue) fully inhibits the binding of antibody to rMOMP, whereas an amino acid change 325(G->A) abolished inhibition (green). Letter height shows the contribution of individual amino acids in the epitopes at 3.12 µM peptide.

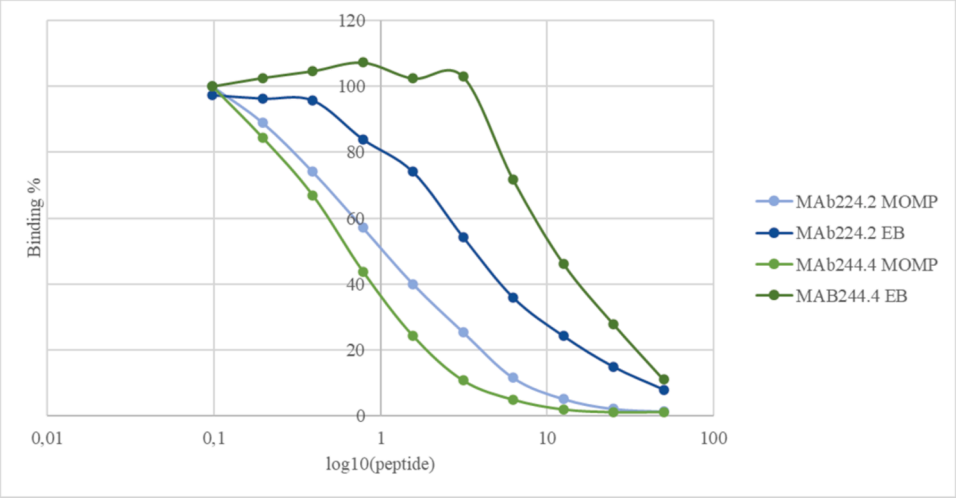

Fig. 5. Competition ELISA. MAb224.2 (blue lines) and MAb244.4 (green lines) binding to either *C. trachomatis* serovar D rMOMP or EB. Synthetic peptides of MOMP VDIV were added in a 2-fold-dilution for competitive inhibition.

genital epithelial cells express neonatal Fc receptor (FcRn) for transcytosis of IgG (Armitage et al., 2014). Therefore, the observed uptake in HeLa cells could be Fc-receptor mediated. To investigate this, HaK cells lacking Fc-receptors were used. Similar results as for HeLa cells were obtained for *C. trachomatis* L2 treated with MAb224 and MAb244.4 (Fig. 7C and D), where red stained EB were seen more central in the cell. For *C. trachomatis* D, more red and green stained chlamydiae were seen due to the centrifugation step, but most were stained red when taken up into the cell (Fig. 7E, F).

4. Discussion

In the present study we characterized the binding affinity of the two monoclonal antibodies, MAb224.2 and MAb244.4, directed against surface exposed MOMP VDIV of *C. trachomatis* and examined their protective contribution. By immunofluorescence microscopy we showed that both MAb224.2 and MAb244.4 reacted with inclusions in *C. trachomatis* D, E, and L2 infected cells (Fig. 1A-I) and immunoelectron microscopy confirmed that the MAb reacted with the unfixed EB surface. Immunogold particles covered the entire surface of EB and demonstrated that MOMP VDIV epitopes are surface exposed (Fig. 1J-L). To further analyze the binding affinity between the two MAb and MOMP we used Real-time surface plasmon resonance which showed that both MAb had high binding affinity (Fig. 3).

We further found that both MAbs mapped the linear peptide sequence of MOMP VDIV ^{322}TIAGAGD^{328} that holds species-specific epitopes. Therefore, we determined the contribution to binding of the individual amino acids in VDIV. This was investigated using competition ELISA which indicated that amino acids ^{325}G and ^{323}I were crucial for binding of the antibodies to the epitope on the chlamydial surface (Fig. 4). This surface-exposed and species-specific epitope could potentially function as candidate for vaccine development. Several studies have found MOMP to be a strong antigen as it is exposed to the humoral immune system. Moreover, MOMP is found to be the immunodominant chlamydial outer membrane protein (Sun et al., 2007).

Next, we examined a conceivable divalent binding to the surface of EB by comparing the binding of MAb224.2 and MAb244.4 to rMOMP or to the EB surface. This was confirmed using competition ELISA, which showed that higher concentrations of competitive peptides were needed to inhibit the binding of MAb244.4 to EB rather than to rMOMP. This points to the fact that MAb244.4 could provide more than a single bond to its antigen and thereby it binds to two VDIV loops on the chlamydial surface simultaneously, resulting in a higher binding avidity (Fig. 5).

Only MAb244.4 with isotype IgG1 could bind divalently to MOMP. This could be caused by the high density of MOMP on the EB surface as seen by IEM, thus the antibody is capable of binding to two MOMP epitopes at the same time. On the other hand, it could also be caused by the different subclasses of IgG that may influence the binding of antibodies. Contrary to MAb244.4, MAb224.2 belongs to the IgG2a subclass, and for each subclass the structures of the antibodies are distinct. Cooper et al. (1994) reported that differences in affinity and kinetic constants of variable domain identical IgG antibodies are related to differences in their antibody subclass. Thus, the structure of constant domains can impact both equilibrium and kinetic constants characterizing interactions of IgG with multivalent antigens. This could be explained by variation of the hinge region among antibodies of different subclasses resulting in different flexibility, and this could explain the difference in avidity of the two antibodies and thereby the divalent binding of MAb244.4 to EB.

Despite the strong binding to the EB surface, the neutralization assay with MAb224.2 and MAb244.4 directed against *C. trachomatis* D MOMP demonstrated a neutralizing effect on inclusion forming units of only 38%. To determine why the MAb against MOMP were unable to reduce the infectivity to more than 38%, we examined entrance of *C. trachomatis* into the host cell in the presence of MAb224.2 and MAb244.4. Uptake of *Chlamydia* into the host cells was not affected by the binding of MAb to the chlamydial surface, thereby indicating that the function of PmpD was not affected, and that also the T3SS system functioned. Though uptake of EB was unaffected by MAb binding, staining by MAb disappeared from the EB surface after entering the cell (Fig. 6). This is an advantage for the further development of the chlamydial inclusion and can be the reason for the missing neutralization of the infection. Factors in the chlamydial inclusion that could cause the loss of MAb staining, could be that the redox potential in the chlamydial inclusion could reduce the disulfide bonds and thereby disintegrating the antibodies. The lack of reduction in infectivity and lack of detection of MOMP antibodies on the EB surface after host cell entry limits MOMPs function as a potential vaccine candidate.

In a study by Collar et al. (2020) they analyzed the production of IgG antibodies against MOMP VDIV in three different groups of women diagnosed with *C. trachomatis*: 1) negative upon treatment (spontaneous resolver), 2) negative at 3-month follow-up (non-reinfected), and 3) positive at 3-month follow-up (reinfected). They found that all three groups produced MOMP-specific antibodies suggesting no correlation between MOMP VDIV antibodies and protection against reinfection (Collar et al., 2020). However, antibodies to MOMP VDIV were the most identified among the patients, supporting its function as an immunodominant B cell epitope during a urogenital infection in women. This

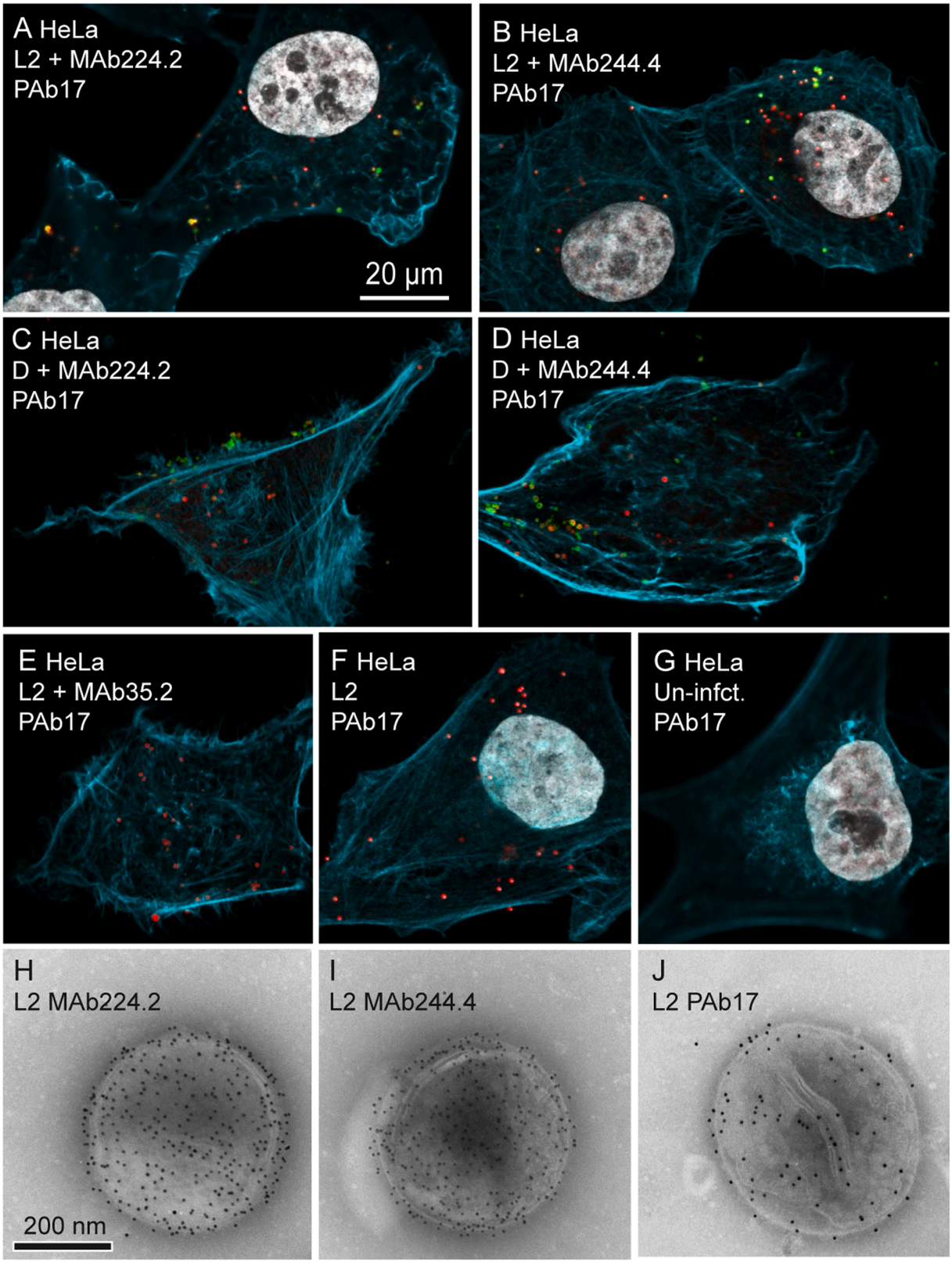

Fig. 6. Immunofluorescence microscopy. MAb224.2, MAb244.4, and MAb35.2 (DnaK) were incubated with *C. trachomatis* L2 (A, B, E) and *C. trachomatis* D (C, D) and added to HeLa cell monolayers for 2 hrs. After incubation, cells were fixed and stained with primary antibody: rabbit PAb17 against *C. trachomatis* L2 COMC. For visualization FITC-conjugated anti-mouse IgG (green), Alexa-647 conjugated anti-rabbit IgG (red), polymeric actin (cyan) and DNA (white) were used. (F) control without MAb and (G) control without *C. trachomatis.* Immunoelectron microscopy of purified *C. trachomatis* L2 EB reacted with MAb224.2 (H), MAb244.4 (I), or PAb17 (J) and visualized by 10 nm immuno gold secondary antibodies, bar 200 nm.

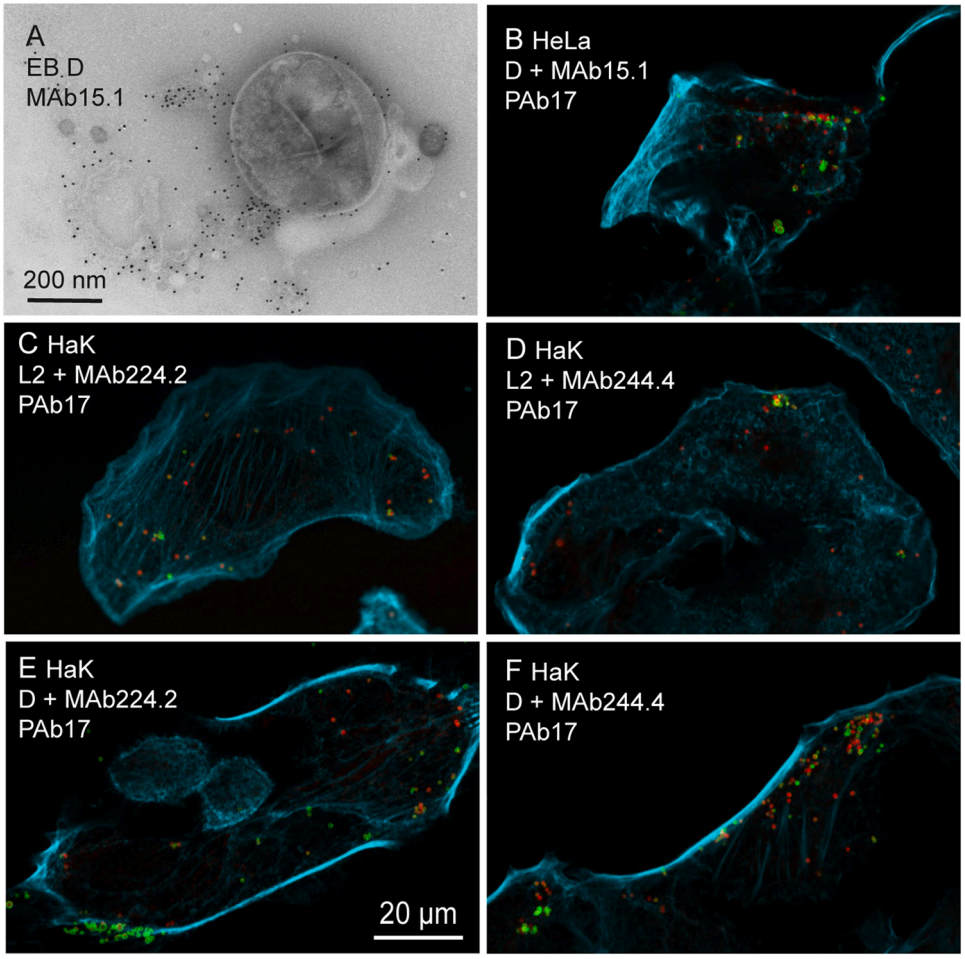

Fig. 7. Immuno microscopy. Immunoelectron microscopy of purified *C. trachomatis* D EB reacted with MAb15.1 against chlamydial LPS visualized by 10 nm immune gold secondary antibodies, bar 200 nm (A). Immunofluorescence microscopy. MAb15.1 were incubated with *C. trachomatis* added to HeLa cell monolayers for 2 hrs (B). MAb224.2 and MAb244.4, were incubated with *C. trachomatis* L2 (C, D) and *C. trachomatis* D (E, F) and added to HaK cell monolayers for 2 hrs. After incubation cells were fixed and stained with primary antibody rabbit PAb17 against *C. trachomatis* L2 COMC. For visualization, FITC-conjugated anti-mouse IgG (green), Alexa-647 conjugated anti-rabbit IgG (red) and polymeric actin (cyan) were used.

finding is supported by our results. The only identified agent correlated with protective effects against infection with *C. trachomatis* is INF-gamma producing $CD4^+$ T-cells, which possibly decreases incidence and reinfection frequency, and is therefore an important element of adaptive immunity to *C. trachomatis* infection (Bakshi et al., 2018). Nevertheless, evidence suggests that targeting immunodominant regions of antigens may not provide protection. Alternatively, identifying epitopes for antibodies inhibiting attachment of *C. trachomatis* to host cells may serve as candidate for vaccine development.

5. Conclusion

In this paper it was shown that antibodies to VDIV MOMP epitope ^{322}TIAGAGD^{328} can bind divalently to the highly ordered rigid surface of *C. trachomatis* EB, and thereby obtain an extremely high avidity. The infectivity was, however, only reduced marginally by the antibodies to VDIV, and the antibodies did not interfere with the uptake into the host cell. After internalization, the antibodies disappeared from EB, and these results may explain both the high immunogenicity of VDIV and why the antibodies do not neutralize the infection.

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CRediT authorship contribution statement

Laura Lind Throne Degn: Conceptualization, Writing – original draft, Writing – review & editing, Visualization. **Ditte Bech:** Methodology. **Gunna Christiansen:** Conceptualization, Writing – review & editing. **Svend Birkelund:** Methodology, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition, Conceptualization, Funding acquisition, Validation, Resources, Writing – review & editing.

Declaration of Competing Interest

None of the authors as competing interest.

Data Availability

Data will be made available on request.

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References

Armitage, C.W., O'meara, C.P., Harvie, M.C.G., Timms, P., Blumberg, R.S., Beagley, K. W., 2014. Divergent outcomes following transcytosis of IgG targeting intracellular and extracellular chlamydial antigens. Immunol. Cell Biol. 92, 417–426. https://doi.org/10.1038/icb.2013.110.

Axelsen, T.V., Holm, A., Birkelund, S., Christiansen, G., Ploug, M., Holm, I.E., 2009. Specific recognition of the C-terminal end of A beta 42 by a high affinity monoclonal antibody. Mol. Immunol. 46, 2267–2273. https://doi.org/10.1016/j.molimm.2009.04.007.

Bakshi, R.K., Gupta, K., Jordan, S.J., Chi, X., Lensing, S.Y., Press, C.G., Geisler, W.M., 2018. An Adaptive Chlamydia trachomatis-specific IFN-γ-producing CD4+ T cell response is associated with protection against Chlamydia reinfection in women. Front Immunol. 9 https://doi.org/10.3389/fimmu.2018.01981.

Birkelund, S., Lundemose, A.G., Christiansen, G., 1988. Chemical cross-linking of Chlamydia trachomatis. Infect. Immun. 56, 654–659. https://doi.org/10.1128/iai.56.3.654-659.1988.

Birkelund, S., Lundemose, A.G., Christiansen, G., 1989. Immunoelectron microscopy of lipopolysaccharide in Chlamydia trachomatis. Infect. Immun. 57, 3250–3253. https://doi.org/10.1128/iai.57.10.3250-3253.1989.

Birkelund, S., Lundemose, A.G., Christiansen, G., 1990. The 75-kilodalton cytoplasmic Chlamydia trachomatis L2 polypeptide is a DnaK-like protein. Infect. Immun. 58 https://doi.org/10.1128/iai.58.7.2098-2104.1990.

Birkelund, S., Johnsen, H., Christiansen, G., 1994a. Chlamydia trachomatis serovar L2 induces protein tyrosine phosphorylation during uptake by HeLa cells. Infect. Immun. 62, 4900–4908. https://doi.org/10.1128/iai.62.11.4900-4908.1994.

Birkelund, S., Larsen, B., Holm, A., Lundemose, A.G., Christiansen, G., 1994b. Characterization of a linear epitope on Chlamydia trachomatis serovar L2 DnaK-like protein. Infect. Immun. 62, 2051–2057.

Brade, H., Brade, L., Nano, F.E., 1987. Chemical and serological investigations on the genus-specific lipopolysaccharide epitope of Chlamydia. Proc. Natl. Acad. Sci. USA 84, 2508–2512. https://doi.org/10.1073/pnas.84.8.2508.

Bumbueviciute, R., Holm, A., Christiansen, G., Birkelund, S. 2008. Characterization of antibody binding to Chlamydia trachomatis major outer membrane protein variable domain IV. In: 12th International symposium on Human Chlamydia Infections. pp. 137-140.

Caldwell, H.D., Kromhout, J., Schachter, J., 1981. Purification and partial characterization of the major outer membrane protein of Chlamydia trachomatis. Infect. Immun. 31 https://doi.org/10.1128/iai.31.3.1161-1176.1981.

Collar, A.L., Linville, A.C., Core, S.B., Wheeler, C.M., Geisler, W.M., Peabody, D.S., Chackerian, B., Frietze, K.M., 2020. Antibodies to variable domain 4 linear epitopes of the chlamydia trachomatis major outer membrane protein are not associated with chlamydia resolution or reinfection in women. mSphere 5. https://doi.org/10.1128/mSphere.00654-20.

Cooper, L.J.N., Robertson, D., Granzow, R., Greenspan, N.S., 1994. Variable domain-identical antibodies exhibit IgG subclass-related differences in affinity and kinetic constants as determined by surface plasmon resonance. Mol. Immunol. 31, 577–584. https://doi.org/10.1016/0161-5890(94)90165-1.

Everett, K.D.E., Desiderio, D.M., Hatch, T.P., 1994. Characterization of lipoprotein EnvA in Chlamydia psittaci 6BC. J. Bacteriol. 176 https://doi.org/10.1128/jb.176.19.6082-6087.1994.

Findlay, H.E., McClafferty, H., Ashley, R.H., 2005. Surface expression, single-channel analysis and membrane topology of recombinant Chlamydia trachomatis Major Outer Membrane Protein. BMC Microbiol 5. https://doi.org/10.1186/1471-2180-5-5.

Gitsels, A., Sanders, N., Vanrompay, D., Dandekar, T., Ball, S.G., 2019. Chlamydial infection from outside to inside, 10, 1–27. https://doi.org/10.3389/fmicb.2019.02329.

Grimwood, J., Stephens, R.S., 1999. Computational analysis of the polymorphic membrane protein superfamily of Chlamydia trachomatis and Chlamydia pneumoniae. Micro Comp. Genom. 4 https://doi.org/10.1089/omi.1.1999.4.187.

Hatch, T.P., Miceli, M., Sublett, J.E., 1986. Synthesis of disulfide-bonded outer membrane proteins during the developmental cycle of Chlamydia psittaci and Chlamydia trachomatis. J. Bacteriol. 165 https://doi.org/10.1128/jb.165.2.379-385.1986.

Hobolt-Pedersen, A.S., Christiansen, G., Timmerman, E., Gevaert, K., Birkelund, S., 2009. Identification of Chlamydia trachomatis CT621, a protein delivered through the type III secretion system to the host cell cytoplasm and nucleus. FEMS Immunol. Med Microbiol 57. https://doi.org/10.1111/j.1574-695X.2009.00581.x.

Jones, H.M., Kubo, A., Stephens, R.S., 2000. Design, expression and functional characterization of a synthetic gene encoding the Chlamydia trachomatis major outer membrane protein. Gene 258. https://doi.org/10.1016/S0378-1119(00)00367-X.

Lausen, M., Thomsen, M.E., Christiansen, G., Karred, N., Stensballe, A., Bennike, T.B., Birkelund, S., 2021. Analysis of complement deposition and processing on Chlamydia trachomatis. Med Microbiol Immunol. 210, 13–32. https://doi.org/10.1007/s00430-020-00695-x.

Murray, S.M., McKay, P.F., 2021. Chlamydia trachomatis: cell biology, immunology and vaccination. Vaccine. https://doi.org/10.1016/j.vaccine.2021.03.043.

Mygind, P., Christiansen, G., Birkelund, S., 1998. Topological analysis of Chlamydia trachomatis L2 outer membrane protein 2. J. Bacteriol. 180 https://doi.org/10.1128/jb.180.21.5784-5787.1998.

Nans, A., Saibil, H.R., Hayward, R.D., 2014. Pathogen-host reorganization during Chlamydia invasion revealed by cryo-electron tomography. Cell Microbiol 16, 1457–1472. https://doi.org/10.1111/cmi.12310.

Nurminen, M., Rietschel, E.T., Brade, H., 1985. Chemical characterization of Chlamydia trachomatis lipopolysaccharide. Infect. Immun. 48, 573–575. https://doi.org/10.1128/iai.48.2.573-575.1985.

Olsen, A.W., Rosenkrands, I., Holland, M.J., Andersen, P., Follmann, F., 2021. A Chlamydia trachomatis VD1-MOMP vaccine elicits cross-neutralizing and protective antibodies against C/C-related complex serovars. NPJ Vaccin. 6 https://doi.org/10.1038/s41541-021-00312-9.

Paes, W., Brown, N., Brzozowski, A.M., Coler, R., Reed, S., Carter, D., Bland, M., Kaye, P. M., Lacey, C.J.N., 2016. Recombinant polymorphic membrane protein D in combination with a novel, second-generation lipid adjuvant protects against intravaginal Chlamydia trachomatis infection in mice. Vaccine 34, 4123–4131. https://doi.org/10.1016/j.vaccine.2016.06.081.

Paes, W., Dowle, A., Coldwell, J., Leech, A., Ganderton, T., Brzozowski, A., 2018. The Chlamydia trachomatis PmpD adhesin forms higher order structures through disulphide-mediated covalent interactions. PLoS One 13, e0198662. https://doi.org/10.1371/journal.pone.0198662.

Shaw, Allan C., Vandahl, B.B., Larsen, M.R., Roepstorff, P., Gevaert, K., Vandekerckhove, J., Christiansen, G., Birkelund, S., 2002a. Characterization of a secreted Chlamydia protease. Cell Microbiol 4, 411–424. https://doi.org/10.1046/j.1462-5822.2002.00200.x.

Shaw, Allan C., Vandahl, B.B., Larsen, M.R., Roepstorff, P., Gevaert, K., Vandekerckhove, J., Christiansen, G., Birkelund, S., 2002b. Characterization of a secreted Chlamydia protease. Cell Microbiol 4, 411–424. https://doi.org/10.1046/j.1462-5822.2002.00200.x.

Stephens, R.S., Tam, M.R., Kuo, C.C., Nowinski, R.C., 1982. Monoclonal antibodies to Chlamydia trachomatis: antibody specificities and antigen characterization. J. Immunol. 128 https://doi.org/10.4049/jimmunol.128.3.1083.

Stephens, R.S., Sanchez-Pescador, R., Wagar, E.A., Inouye, C., Urdea, M.S., 1987. Diversity of Chlamydia trachomatis major outer membrane protein genes. J. Bacteriol. 169 https://doi.org/10.1128/jb.169.9.3879-3885.1987.

Stephens, R.S., Wagar, E.A., Schoolnik, G.K., 1988. High-resolution mapping of serovar-specific and common antigenic determinants of the major outer membrane protein of chlamydia trachomatis. J. Exp. Med. 167 https://doi.org/10.1084/jem.167.3.817.

Su, H., Spangrude, G.J., Caldwell, H.D., 1991. Expression of FcγRIII on HeLa 229 cells: possible effect on in vitro neutralization of Chlamydia trachomatis. Infect. Immun. https://doi.org/10.1128/iai.59.10.3811-3814.1991.

Sun, G., Pal, S., Sarcon, A.K., Kim, S., Sugawara, E., Nikaido, H., Cocco, M.J., Peterson, E. M., De La Maza, L.M., 2007. Structural and functional analyses of the major outer membrane protein of Chlamydia trachomatis. J. Bacteriol. 189 https://doi.org/10.1128/JB.00552-07.

Vasilevsky, S., Greub, G., Nardelli-Haefliger, D., Baud, D., 2014. Genital Chlamydia trachomatis: understanding the roles of innate and adaptive immunity in vaccine research. Clin. Microbiol Rev. 27 https://doi.org/10.1128/CMR.00105-13.

Vasilevsky, S., Stojanov, M., Greub, G., Baud, D., 2016. Chlamydial polymorphic membrane proteins: regulation, function and potential vaccine candidates. Virulence. https://doi.org/10.1080/21505594.2015.1111509.

Wang, S., Grayston, J.T., 1974. Human serology in Chlamydia trachomatis infection with microimmunofluorescence. J. Infect. Dis. 130 https://doi.org/10.1093/infdis/130.4.388.

Wyllie, S., Ashley, R.H., Longbottom, D., Herring, A.J., 1998. The major outer membrane protein of Chlamydia psittaci functions as a porin-like ion channel. Infect. Immun. 66 https://doi.org/10.1128/iai.66.11.5202-5207.1998.