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### Title

Co-delivery of amphipol-conjugated adjuvant with antigen, and adjuvant combinations, enhance immune protection elicited by a membrane protein-based vaccine against a mucosal challenge with Chlamydia

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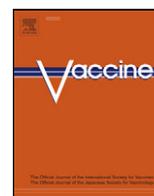
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## Amphipols stabilize the *Chlamydia* major outer membrane protein and enhance its protective ability as a vaccine

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### ABSTRACT

The native major outer membrane protein (nMOMP) from *Chlamydia* was purified in its trimeric form using the zwitterionic detergent Z3-14. In aliquots from this preparation, Z3-14 was exchanged for amphipol (APol) A8-35. CD analysis showed that trapping with A8-35 improved the thermostability of nMOMP without affecting its secondary structure. Recombinant MOMP (rMOMP) was also formulated with Z3-14 or A8-35. Four groups of mice were vaccinated with nMOMP/Z3-14, nMOMP/A8-35, rMOMP/Z3-14 or rMOMP/A8-35 using CpG and Montanide as adjuvants. A positive control group was inoculated intranasally with live *Chlamydia* and a negative control group with culture medium. Mice were challenged intranasally with live *Chlamydia* and protection was assessed based on changes in body weight, the weight of the lungs and the number of chlamydial inclusion forming units recovered from the lungs 10 days after the challenge. Overall, vaccines formulated with nMOMP elicited better protection than those using rMOMP. Furthermore, the protection afforded by nMOMP/A8-35 was more robust than that achieved with nMOMP/Z3-14. In contrast, no differences in protection were observed between rMOMP/Z3-14 and rMOMP/A8-35 preparations. These findings suggest that the higher protection conferred by nMOMP/A8-35 complexes most likely results from a better preservation of the native structure of MOMP and/or from a more efficient presentation of the antigen to the immune system, rather than from an adjuvant effect of the amphipol. Thus, amphipols can be used in vaccine formulations to stabilize a membrane-protein component and enhance its immunogenicity.

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### 1. Introduction

*Chlamydia trachomatis* is one of the most common bacterial pathogens found in all regions of the World [1]. Infections with this organism can affect persons of all ages. In young individuals, *C. trachomatis* is the most common sexually transmitted bacterial pathogen [2,3]. Genital infections can remain asymptomatic but others can produce acute symptomatology. In women, long-term sequelae such as infertility and ectopic pregnancy can develop [4]. At birth, newborns can become infected in the eyes and lungs if the mother has a genital tract infection at the time of delivery [5,6]. *C. trachomatis* has also been isolated from the lungs of adults, in particular from immunocompromised patients [7,8]. In countries with poor hygienic conditions, young children can have multiple ocular infections that result in the development of trachoma later on in life [3,9–11]. In addition, the lymphogranuloma venereum serovars

of *C. trachomatis* can produce severe medical complications due to scarring and stenosis of the lymphatics [3,12]. Antibiotic therapy is available for chlamydial infections but many individuals go untreated and even patients that are treated may develop chronic sequelae when this pathogen establishes a persistent infection [13].

Attempts to produce a vaccine against *C. trachomatis* were initiated in the 1960s [3,9,10,14]. Vaccines formulated with whole inactivated and viable organisms were tested in humans and in non-human primates to protect against trachoma. Several conclusions were reached from those studies [3,9,14]. Some vaccine protocols induced protection, but the latter lasted only 1–2 years. In addition, it appeared to be serovar specific, i.e., of the four *C. trachomatis* ocular isolates, A, B, Ba and C, the protection was effective only against the serovar used in the vaccine. Furthermore, after reexposure to *Chlamydia*, some of the immunized individuals developed a hypersensitivity reaction. Although the cause of the hypersensitivity reaction has not yet been elucidated, it is attributed to an antigenic component present in *Chlamydia* [15]. Therefore, the need to develop a subunit vaccine was considered.

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The *C. trachomatis* major outer membrane protein (MOMP) belongs to a family of proteins found in the outer membrane of Gram-negative bacteria whose monomers have a molecular mass of ~40 kDa and the homotrimers function as porins [16,17]. DNA sequencing of *C. trachomatis* MOMP identified four variable domains (VDs) that are unique to each serovar and, therefore, most likely account for the serovar-specific protection observed during the trachoma trials [9,18]. SDS-PAGE analyses of native MOMP (nMOMP) purified from *C. trachomatis* mouse pneumonitis (MoPn) using Z3-14, a zwitterionic detergent, showed it to be a homotrimer [16]. A topological model of MoPn MOMP proposes that each monomer comprises 16 antiparallel  $\beta$ -strands that form a barrel structure spanning the outer membrane while the VDs are exposed to the surface [19]. Pal et al. immunized mice with the *C. trachomatis* MoPn nMOMP formulated with the detergent Z3-14, challenged them intranasally or in the genital tract and observed a significant protective immune response [20,21]. Furthermore, Kari et al. [22] showed that monkeys immunized with a similar nMOMP preparation were significantly protected against an ocular challenge with *C. trachomatis* serovar A.

In general, integral membrane proteins are kept soluble in aqueous solutions using detergents. In 1996, Tribet et al. [23] introduced amphipathic polymers called amphipols (APols). APols were designed to keep membrane proteins soluble in water in the absence of free surfactants, while stabilizing them biochemically. APols have been shown to keep soluble, in their native conformation, about two-dozen integral membrane proteins, including matrix porin (OmpF) from *Escherichia coli*, a protein whose structure is similar to that of *C. trachomatis* MOMP [24,25]. In the present study, we extracted nMOMP, using Z3-14, and then exchanged this detergent for A8-35. In addition, we prepared rMOMP and kept it soluble using either Z3-14 or A8-35. Four groups of mice were immunized using nMOMP or rMOMP formulated with either surfactant. As compared to the other three groups of animals, mice vaccinated with nMOMP/A8-35 showed a significant increase in protection against an intranasal challenge with *Chlamydia*.

## 2. Materials and methods

### 2.1. Stocks of *C. trachomatis*

The *C. trachomatis* MoPn strain Nigg II (also called *Chlamydia muridarum*; obtained from the American Type Culture Collection, ATCC; Manassas, VA) was grown in McCoy cells and elementary bodies (EBs) were purified as described and stored in SPG (0.2 M sucrose, 20 mM sodium phosphate pH 7.2 and 5 mM glutamic acid) [26,27].

### 2.2. Purification and preparation of *C. trachomatis* nMOMP and rMOMP

The extraction and purification of nMOMP and rMOMP has been described [28]. Mass spectrometry analyses and N-terminal sequencing of nMOMP both revealed a purity of >99% [29,30]. Using the limulus amoebocyte assay (BioWhittaker, Inc., Walkersville, MD), rMOMP and nMOMP were found to have less than 0.05 EU of LPS/mg of protein [28]. To prepare nMOMP/Z3-14 for immunization, the protein was concentrated and fixed with 2% glutaraldehyde (Sigma-Aldrich, St. Louis, MO) at room temperature for 2 min. Glycine (Bio-Rad Laboratories; Hercules, CA) was added to stop the reaction. nMOMP/Z3-14 was dialyzed against PBS (pH 7.4) with 0.05% Z3-14 before immunization. A8-35 was synthesized as described [31,32]. To formulate nMOMP/A8-35, unfixed nMOMP/Z3-14 at a concentration of 1 mg/ml was incubated at room temperature for 2 h with A8-35 at an APol/MOMP weight ratio

of 2/1. Following incubation, rehydrated BioBeads SM-2 Adsorbent (Bio-Rad) were added to remove Z3-14 at a weight ratio of 1(Z3-14)/2.5  $\times$  10<sup>3</sup> (BioBeads). rMOMP/Z3-14 was dialyzed against PBS (pH 7.4) with 0.05% Z3-14 before immunization. To prepare rMOMP/A8-35, the protein was incubated at room temperature for 2 h with A8-35 at an APol/MOMP weight ratio of 4/1. Following incubation, rehydrated BioBeads SM-2 Adsorbent (BioRad) were added and the mixture incubated at 4 °C for 16 h and then the beads were removed by centrifugation.

### 2.3. Circular dichroism (CD)

Spectra were obtained using a Jasco J-720 spectropolarimeter (Easton, MD) equipped with a Peltier cooling system. The nMOMP samples at 0.033 mg/ml in a solution containing 20 mM NaP<sub>i</sub> pH 7.4, and either 0.05% Z3-14 or A8-35 were analyzed in a 1-mm path length sealed quartz cuvette (Hellma; Mullheim, Germany). Samples were allowed to equilibrate at 24 °C for 5 min, and then subjected to a temperature ramp from 24 to 78 °C. Spectra were obtained at 2 °C intervals, allowing 2 min of temperature equilibration after each increment. Data was acquired in 1 nm increments at 50 nm/min at a 1 nm resolution from 240 to 190 nm. At each temperature, 5 sample scans were acquired and averaged, corrected for baseline distortion by subtracting appropriate reference spectra (average of 3 scans per temperature) and smoothed. Processed spectra are shown in  $\Delta\epsilon$  units (mDeg/M cm). Two replicates of each sample were analyzed by CD. The spectra shown are from an individual experiment, while the plot showing the value of  $\Delta\epsilon$  at 208 nm as a function of temperature represents the average of two experiments.

### 2.4. SDS-PAGE analysis

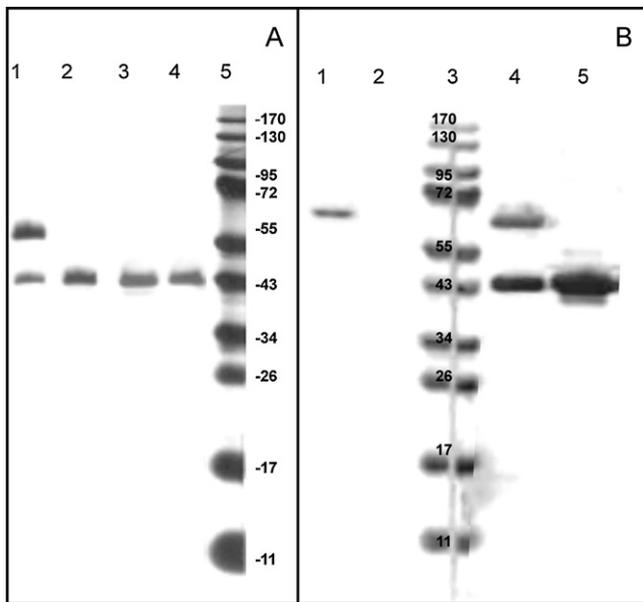
The apparent MW and purity of nMOMP and rMOMP were determined by 10% tricine-SDS-PAGE [33].

### 2.5. Immunization protocols

Three-week-old female BALB/c (H-2<sup>d</sup>) mice were purchased from Charles River Laboratories (Wilmington, MA). Animals were vaccinated with nMOMP and rMOMP by the intramuscular (i.m.) (7  $\mu$ g protein/mouse/immunization) and the subcutaneous (s.c.) (3  $\mu$ g protein/mouse/immunization) routes. CpG-1826 (10  $\mu$ g/mouse/dose; 5'-TCCATGACGTTCTGACGTT-3'; Coley Pharmaceutical Group, Ontario, Canada) and Montanide ISA 720 (Seppic Inc., Fairfield, NJ) at a 30:70 volume ratio of MOMP plus CpG to Montanide ISA 720 were used as adjuvants [28]. The mice were boosted two times at 2-week intervals with the same vaccine formulation. A negative control group of mice was inoculated intranasally (i.n.) with MEM-0. Positive control mice were immunized i.n. once with 1  $\times$  10<sup>4</sup> inclusion forming units (IFU) of *C. trachomatis* MoPn [28]. All experiments were repeated twice. The animal protocols were approved by the University of California Irvine, Animal Care and Use Committee.

### 2.6. Immunological assays

Blood was collected from the periorbital plexus or from the heart and serum pooled from all the animals of each group. *C. trachomatis* MoPn-specific antibody levels were determined using an ELISA as described [34]. In vitro neutralization assays were performed as published by Peterson et al. [35]. Western and dot blots were performed using nitrocellulose membranes as previously described [34]. A T cell lymphoproliferative assay (LPA) was performed using splenocytes as formerly published [34]. Levels of IFN- $\gamma$  and



**Fig. 1.** (A) SDS-PAGE of the *Chlamydia* nMOMP and rMOMP. Lanes: (1) not heat-denatured nMOMP; (2) heat-denatured nMOMP; (3) not heat-denatured rMOMP; (4) heat-denatured rMOMP; (5) MW standards. (B) Western blot of not heat-denatured *Chlamydia* nMOMP and rMOMP. Lanes: (1) nMOMP probed with mAb MoPn-18b; (2) rMOMP probed with mAb MoPn-18b; (3) MW standards; (4) nMOMP probed with mAb MoPn-40; (5) rMOMP probed with mAb MoPn-40.

IL-6 were determined using commercial kits (BD Pharmingen, San Diego, CA) in supernatants from the stimulated splenic T cells [28].

### 2.7. Intranasal challenge

Four weeks after the last immunization, anesthetized BALB/c mice were challenged i.n. with  $10^4$  IFU of *C. trachomatis* MoPn in 20  $\mu$ l of MEM-0 [34]. Following the i.n. challenge each mouse was weighed daily. On day 10 post-challenge the animals were euthanized and their lungs harvested and weighed. After homogenization in SPG, serial 10-fold dilutions of the tissues were inoculated onto HeLa cells and the *Chlamydia* inclusions were stained with a pool of monoclonal antibodies (mAb) [34].

### 2.8. Statistics

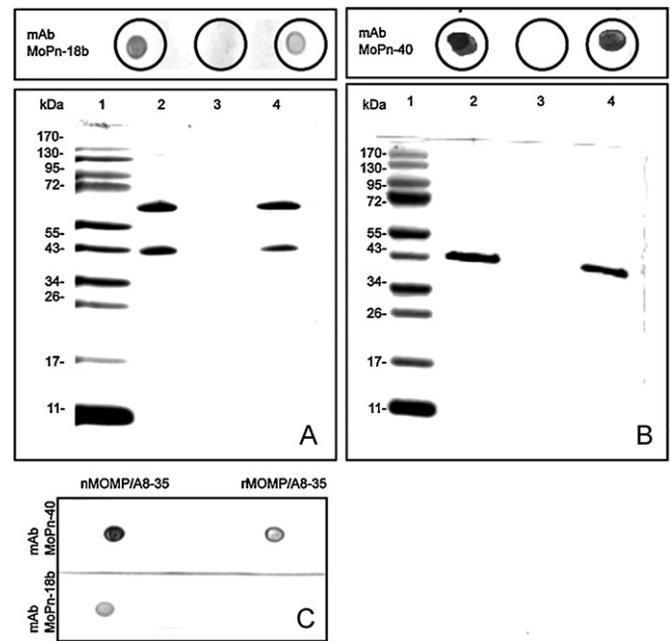
The two-tailed unpaired Student's *t* test, the two-way repeated Anova measures and the Mann–Whitney *U* test were employed to determine the significance of the differences between groups. Differences were considered significant for values of  $P < 0.05$ .

## 3. Results

### 3.1. Characterization of nMOMP and rMOMP preparations by SDS-PAGE and blots

Following extraction and purification the nMOMP and rMOMP were analyzed by SDS-PAGE and stained by Coomassie blue. As shown in Fig. 1A, preparations of rMOMP and nMOMP that were heated before loading migrated as a single band with an apparent molecular mass  $M_r \sim 40$  kDa. When nMOMP preparations were not heated before loading the gel, two bands were observed. The predominant band corresponds to the trimer ( $M_r \sim 66$  kDa) and the second band represents the monomer ( $\sim 40$  kDa). The unheated rMOMP migrated as a monomer.

nMOMP and rMOMP were tested with two mAb MoPn-18b which recognizes the trimeric structure of MoPn MOMP and



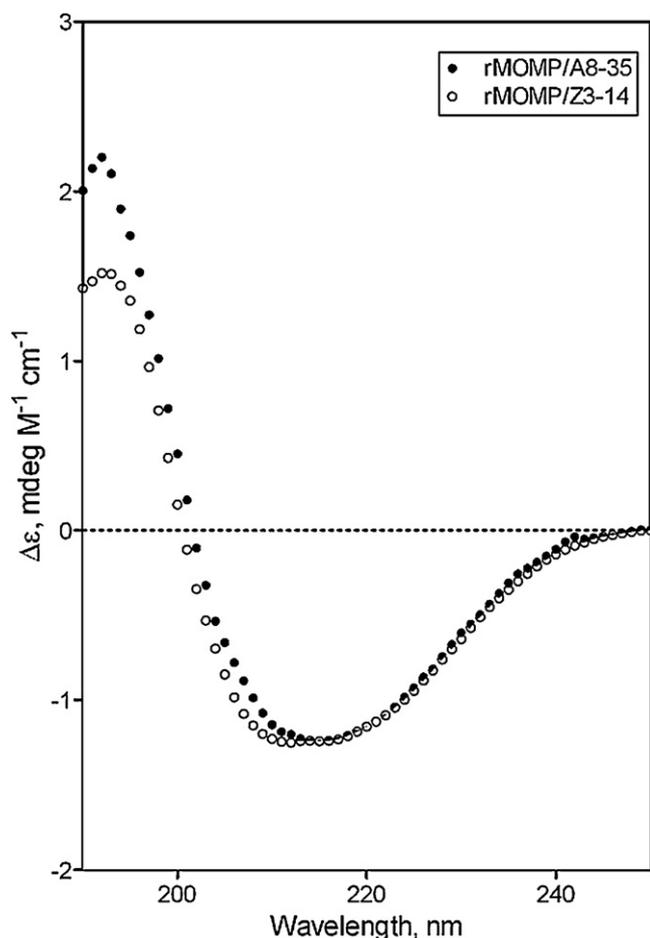
**Fig. 2.** SDS-PAGE of *Chlamydia* nMOMP. Lanes: (1) MW standards; (2) nMOMP/A8-35 following removal of Z3-14 with BioBeads in the presence of A8-35; (3) nMOMP following addition of BioBeads to remove Z3-14 in the absence of A8-35; (4) nMOMP/Z3-14. Top of the figure shows dot blots of same preparations probed with mAb MoPn-18b. (B) SDS-PAGE of *Chlamydia* rMOMP. Lanes: (1) MW standards; (2) rMOMP/A8-35 following removal of Z3-14 with BioBeads in the presence of A8-35; (3) rMOMP following addition of BioBeads to remove Z3-14 in the absence of A8-35; (4) rMOMP/Z3-14. Top of the figure shows dot blots of same preparations probed with mAb MoPn-40. (C) Dot blots of not heat-denatured *Chlamydia* nMOMP/A8-35 and rMOMP/A8-35. Preparations of nMOMP/A8-35 and rMOMP/A8-35 probed with mAbs MoPn-40 (top) and MoPn-18b (bottom).

MoPn-40, which binds to a linear epitope in the VD1 of MOMP. As shown in Fig. 1B, MoPn-18b only recognizes the nMOMP trimer, whereas MoPn-40 recognizes the trimer and monomer of nMOMP and the monomer of rMOMP.

Following purification of MOMP with Z3-14, the detergent was exchanged for APols. The preparations were supplemented with A8-35 and the detergent removed by adsorption onto BioBeads. As a control, BioBeads were added to Z3-14 solubilized MOMP in the absence of A8-35. Following centrifugation the preparations were characterized by dot and Western blots and circular dichroism (CD). As shown in Fig. 2A and B, both Z3-14 and A8-35 were able to maintain nMOMP and rMOMP in solution while both preparations precipitated if the detergent was removed in the absence of APols. These experiments indicate, indirectly, that both Z3-14 and A8-35 interact with nMOMP and rMOMP.

### 3.2. Characterization of the thermal stability of nMOMP by circular dichroism (CD)

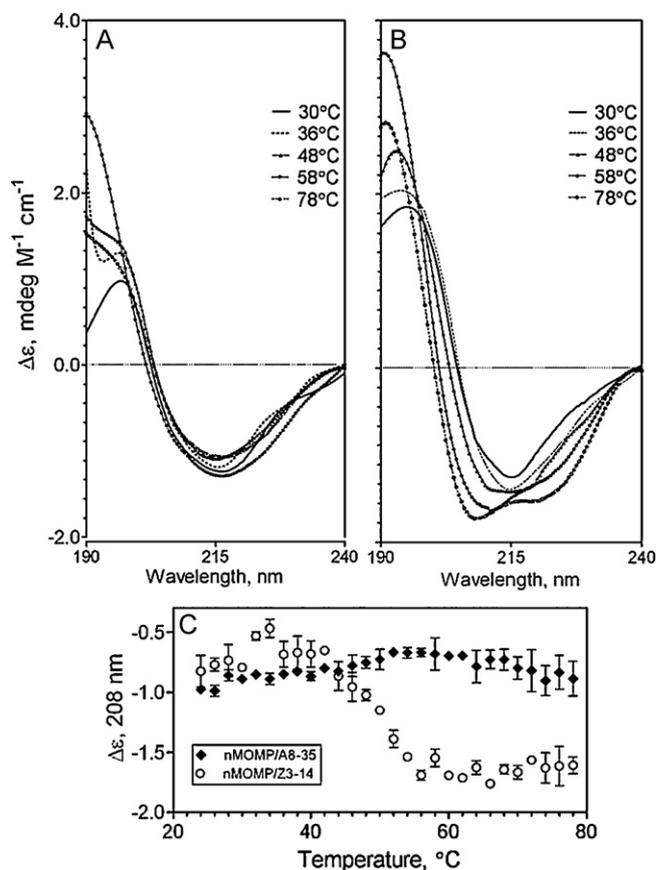
Far-UV CD spectra show evidence of secondary structure with a negative flat peak from  $\sim 220$  to 212 nm for rMOMP/A8-35 and a similar peak from  $\sim 222$  to 208 nm for rMOMP/Z3-14; both spectra show strong positive signals at 195 nm (Fig. 3). These spectra are consistent with a mixture of mostly  $\beta$ -strand and a small amount of  $\alpha$ -helix and disordered structure. The stability of the secondary structure of nMOMP in the presence of either Z3-14 or A8-35 was examined by monitoring changes in the CD spectrum, as function of temperature (Fig. 4). At 24  $^{\circ}$ C, the CD spectra of the two types of complexes were indistinguishable. The CD spectrum of nMOMP/Z3-14 changed with temperature indicating a melting of  $\beta$ -strand and a folding transition to  $\alpha$ -helix. Thus, when nMOMP is solubilized in detergents, we find a melting transition of 52  $^{\circ}$ C;



**Fig. 3.** Circular dichroism spectra of rMOMP/A8-35 and rMOMP/Z3-14. Far-UV spectra of rMOMP with either solubilization agent are shown as  $\Delta\epsilon$  vs. wavelength (250–190 nm).

this value is consistent with published data [36]. Other stable  $\beta$ -strand proteins have been shown to become helical upon thermal denaturation in the presence of detergents [37]. In contrast to the detergent melting, the secondary structure of nMOMP/A8-35 was not visibly affected throughout the temperature range. Thus, amphipols stabilize the protein up to 78 °C. The CD data indicate that the native secondary structure of nMOMP is strongly stabilized when the protein is transferred from Z3-14 to A8-35.

Antibody binding provides additional information on protein conformation and accessibility. Dot blots performed on nMOMP/A8-35 and rMOMP/A8-35 complexes that had not been exposed to SDS, showed that the conformation-sensitive mAb MoPn-18b recognized only nMOMP, while mAb MoPn-40 recognized both nMOMP and rMOMP (Fig. 2C). This indicates that, in keeping with observations on other membrane proteins [38],



**Fig. 4.** Circular dichroism spectra of nMOMP/A8-35 and nMOMP/Z3-14. Preparations of nMOMP (0.033 mg/ml) in 20 mM sodium phosphate, pH 7.4, were incubated at temperatures ranging from 24 to 78 °C and analyzed by CD from 240 to 190 nm. Shown are spectra of (A) nMOMP/A8-35 and (B) nMOMP/Z3-14 in 0.05% Z3-14 at 30, 36, 48, 58 and 78 °C; and (C) the CD units ( $\Delta\epsilon$ ) at 208 nm vs. temperature for each nMOMP preparation.

trapping by A8-35 leaves MOMP's water-exposed surfaces accessible to recognition by antibodies.

### 3.3. Antibody responses in serum

Groups of BALB/c mice were immunized i.m.+s.c. with nMOMP/Z3-14, nMOMP/A8-35, rMOMP/Z3-14 and rMOMP/A8-35, using CpG-1826 and Montanide 720 as adjuvants. As a positive control mice were immunized i.n. with live *Chlamydia* and as a negative control a group of animals received culture medium (MEM-0) i.n. Serum samples were collected the day before the i.n. challenge with *Chlamydia*. As shown in Table 1, both groups of animals immunized with nMOMP had high *Chlamydia*-specific IgG titers (204,800), whereas mice immunized with rMOMP/Z3-14 or rMOMP/A8-35 had IgG antibody titers of 25,600 and 102,400, respectively. The control group immunized i.n. with  $10^4$  IFU of *C. trachomatis* MoPn

**Table 1**  
Anti-*C. trachomatis* MoPn antibody titers in serum from the day before the i.n. challenge.

Antigen	<i>C. trachomatis</i> MoPn specific ELISA antibody titer				Serum neutralizing titer
	IgG	IgG1	IgG2a	Ig	
nMOMP/Z3-14	204,800	12,800	102,400	800	250
nMOMP/A8-35	204,800	51,200	204,800	1600	1250
rMOMP/Z3-14	25,600	1600	25,600	200	250
rMOMP/A8-35	102,400	6400	6400	200	50
CT MoPn	12,800	1600	12,800	1600	1250
MEM-0	<100	<100	<100	<100	<50

had an IgG titer of 12,800. The sera from the negative control group inoculated with MEM-0 contained no chlamydial-specific antibodies.

To determine if the immunizations induced a Th1 or a Th2 response, the titers of IgG1 and IgG2a were measured (Table 1). In the two groups of mice immunized with either nMOMP or rMOMP solubilized in Z3-14 the ratios of IgG2a to IgG1 were 8:1 and 16:1 respectively, indicating a strongly Th1-biased response. This ratio was 4:1, 1:1 and 8:1, respectively, for the groups of mice immunized with nMOMP/A8-35, rMOMP/A8-35 or live EBs.

IgA titers in serum were high in the mice immunized with nMOMP/A8-35 (1600) and nMOMP/Z3-14 (800) and in the positive control group inoculated i.n. with live *Chlamydia* (1600). Both groups of animals vaccinated with rMOMP had low IgA levels in serum (200).

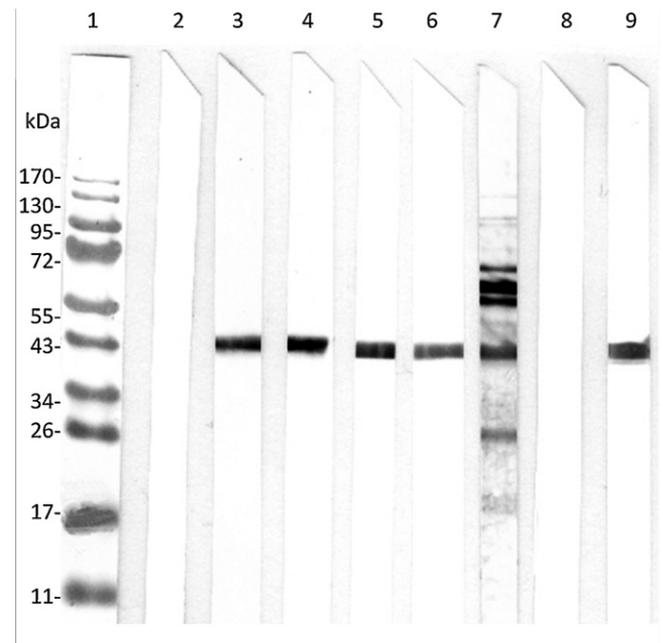
Higher titers of neutralizing antibodies were found in sera from mice vaccinated with nMOMP/Z3-14 (250), nMOMP/A8-35 (1250), or rMOMP/Z3-14 (250) than in that from those immunized with rMOMP/A8-35 (50). The neutralizing titer in the sera from control mice immunized with live *Chlamydia* was 1250. Serum samples from the group inoculated with MEM-0 were used as controls.

### 3.4. Western blot analyses of serum samples

Immunoblot analyses of serum samples, using EBs as the antigen, are shown in Fig. 5. The mice immunized with nMOMP or rMOMP solubilized with either Z3-14 or A8-35 had antibodies only against MOMP. Mice immunized i.n. with live EBs developed antibodies against several antigens above the 100 kDa range, the 60 kDa cysteine rich protein (crp), the 60 kDa heat-shock protein (Hsp), MOMP, the 28 kDa and 18 kDa proteins. No antibodies against chlamydial components were detected in the serum of mice inoculated with MEM-0.

### 3.5. T-cell responses of immunized mice

To determine the T-cell response elicited by vaccination, groups of immunized mice were euthanized and their spleens collected the day before the rest of the animals received the i.n. challenge. T-cells were purified and stimulated with EBs, MEM-0 as a negative control and ConA as a positive control. Proliferation was determined from the incorporation of [<sup>3</sup>H]thymidine. As shown in Table 2, in all groups of animals vaccinated with nMOMP and rMOMP a significant proliferative T-cell immune response was observed ( $P < 0.05$ ). The group immunized with nMOMP/A8-35 showed the highest stimulation index (SI = 90), while that vaccinated with nMOMP/Z3-14 had the lowest (SI = 36). The positive control group showed a



**Fig. 5.** Western blot analyses of serum samples collected the day before the i.n. challenge. Lanes: (1) MW standards. EB probed with (2) pre-immunization sera; Sera from mice immunized with (3) nMOMP/Z3-14; (4) nMOMP/A8-35; (5) rMOMP/Z3-14; (6) rMOMP/A8-35; (7) EB; (8) MEM-0; (9) control mAb MoPn-40.

robust response and the negative control group did not show any significant proliferative T-cell response to EBs.

Levels of IFN- $\gamma$  and IL-6 were determined in the supernatants from splenocytes stimulated with EBs (Table 2). Significant levels of IFN- $\gamma$  were found in the four groups vaccinated with the MOMP preparations. Of these, the highest level of IFN- $\gamma$  was found in the animals immunized with nMOMP/A8-35 (51 ng/ml) and the lowest in the mice vaccinated with rMOMP/Z3-14 (3.6 ng/ml). No significant levels of IL-6 were observed in any of the groups.

### 3.6. Body weight changes following the intranasal challenge

The body weight of the mice was measured daily following the i.n. challenge and was used as an indication of the systemic effect of a chlamydial infection. As shown in Fig. 6, the positive control mice immunized with live EBs lost weight in the first three days following the challenge and then quickly recovered their initial body weight. In contrast, the negative control group inoculated with MEM-0 lost almost 25% of their body weight by 10 days p.i.

**Table 2**

T-cell proliferative responses of immunized mice from the day before the i.n. challenge with *C. trachomatis* MoPn.

Vaccine	T-cell proliferative response <sup>a</sup>				In vitro cytokine production <sup>a</sup>	
	$\times 10^3$ CPM $\pm$ 1 SE to			SI	IFN- $\gamma$ (ng/ml)	IL-6 (ng/ml)
	EB <sup>b</sup>	ConA <sup>c</sup>	Medium		EB <sup>b</sup>	EB <sup>b</sup>
nMOMP/Z3-14	6.59 $\pm$ 0.9 <sup>d,e</sup>	35.1 $\pm$ 2.3	0.18 $\pm$ 0.0	36.6	10.0 $\pm$ 2.1 <sup>d</sup>	0.47 $\pm$ 0.08
nMOMP/A8-35	18.01 $\pm$ 2.4 <sup>d,g</sup>	44.2 $\pm$ 3.2	0.20 $\pm$ 3.2	90.0	51.6 $\pm$ 12.7 <sup>d,g</sup>	1.9 $\pm$ 0.3
rMOMP/Z3-14	4.47 $\pm$ 0.7 <sup>d,f</sup>	44.8 $\pm$ 4.5	0.11 $\pm$ 0.0	40.6	3.6 $\pm$ 0.4 <sup>d</sup>	0.5 $\pm$ 0.09
rMOMP/A8-35	9.85 $\pm$ 0.9 <sup>d</sup>	40.1 $\pm$ 4.3	0.18 $\pm$ 0.0	54.7	35.0 $\pm$ 1.0 <sup>d,g</sup>	0.7 $\pm$ 0.08
CT MoPn	15.13 $\pm$ 1.6 <sup>d</sup>	40.9 $\pm$ 3.2	0.13 $\pm$ 0.0	116.3	131.3 $\pm$ 70.6 <sup>d</sup>	0.9 $\pm$ 0.1
MEM-0	0.46 $\pm$ 0.1	41.3 $\pm$ 4.7	0.08 $\pm$ 0.0	5.7	0.6 $\pm$ 0.02	0.4 $\pm$ 0.1

<sup>a</sup> Values are indicated as means  $\pm$  1 SE of triplicate cultures.

<sup>b</sup> *C. trachomatis* MoPn EB were added at a ratio of 10:1 to APC+T cells.

<sup>c</sup> Concanalin A was added at a concentration of 5  $\mu$ g/ml.

<sup>d</sup>  $P < 0.05$  by Student's *t* test compared to MEM-0 immunized group.

<sup>e</sup>  $P < 0.05$  by Student's *t* test compared to nMOMP/A8-35 immunized group.

<sup>f</sup>  $P < 0.05$  by Student's *t* test compared to rMOMP/A8-35 immunized group.

<sup>g</sup>  $P > 0.05$  by Student's *t* test compared to CT MoPn immunized group. SI, stimulation index (EB/medium).

**Table 3**  
Disease burden and yields of *C. trachomatis* from the lungs.

Antigen	% Change in body weight <sup>a</sup> (mean ± 1 SE)	Lung weight (g) <sup>a</sup> (mean ± 1 SE)	No. IFU recovered from lungs median (min–max), × 10 <sup>3b</sup>
nMOMP/Z3-14	−1.32 ± 0.67 <sup>c,d</sup>	0.24 ± 0.01 <sup>c,d</sup>	140 (5–2333) <sup>h,i,j</sup>
nMOMP/A8-35	0.58 ± 0.88 <sup>c,e,f</sup>	0.23 ± 0.01 <sup>c,e,f</sup>	0.2 (<0.05–3932) <sup>i,j</sup>
rMOMP/Z3-14	−6.03 ± 1.59 <sup>c</sup>	0.29 ± 0.02 <sup>c,g</sup>	13,938 (159–2,003,050) <sup>i,j,k</sup>
rMOMP/A8-35	−6.17 ± 2.33 <sup>c</sup>	0.28 ± 0.01 <sup>c,g</sup>	5492 (186–3,531,150) <sup>i,j</sup>
CT MoPn	1.55 ± 0.76 <sup>c</sup>	0.24 ± 0.01 <sup>c</sup>	<0.05 (<0.05–0.3) <sup>j</sup>
MEM 0	−24.78 ± 0.73	0.36 ± 0.01	4,718,525 (681,450–23,460,000) <sup>j</sup>

<sup>a</sup> Values are indicated as mean ± 1 SE.

<sup>b</sup> Values are indicated as medians and range (minimum–maximum).

<sup>c</sup>  $P < 0.05$  by Student's *t* test compared to MEM-O immunized group.

<sup>d</sup>  $P < 0.05$  by Student's *t* test compared to nMOMP/A8-35 immunized group.

<sup>e</sup>  $P > 0.05$  by Student's *t* test compared to CT MoPn immunized group.

<sup>f</sup>  $P < 0.05$  by Student's *t* test compared to rMOMP/A8-35 immunized group.

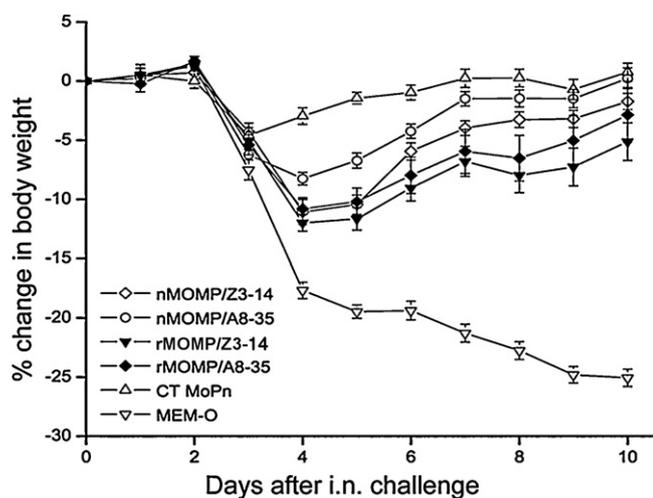
<sup>g</sup>  $P < 0.05$  by Student's *t* test compared to CT MoPn immunized group.

<sup>h</sup>  $P < 0.05$  by Mann–Whitney Rank test compared to nMOMP/A8-35 immunized group.

<sup>i</sup>  $P < 0.05$  by Mann–Whitney Rank test compared to CT MoPn immunized group.

<sup>j</sup>  $P < 0.05$  by Mann–Whitney Rank test compared to MEM-O immunized group.

<sup>k</sup>  $P > 0.05$  by Mann–Whitney Rank test compared to rMOMP/A8-35 immunized group.

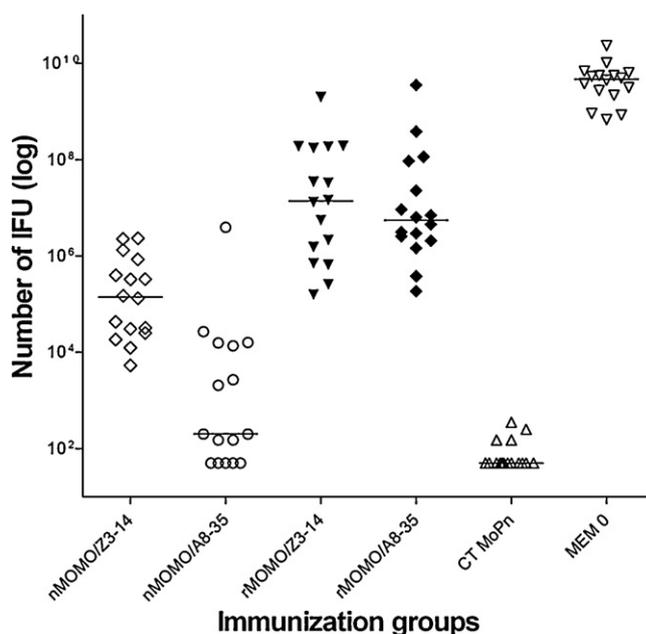


**Fig. 6.** Percentage change in mean body weight of mice following the i.n. challenge with *C. trachomatis* MoPn.

( $P < 0.05$ ). By the 4th day after the i.n. challenge, the group vaccinated with nMOMP/A8-35 had lost 8.25% of their body weight. This group of mice quickly recovered most of their weight and by day 10 after the challenge the mean body weight was 0.58% above its initial value. A similar pattern was observed for mice vaccinated either with nMOMP/Z3-14 or with any of the two rMOMP preparations, but in all three cases the initial weight loss was more severe and the recovery slower than for mice that had received nMOMP/A8-35. At 10 days post-challenge, all groups of mice vaccinated with nMOMP or rMOMP had lost significantly less weight than the group inoculated with MEM-0 ( $P < 0.05$ ) as shown in Table 3 and Fig. S1.

### 3.7. Weight of the lungs

At 10 days following the i.n. challenge the mice were euthanized and their lungs harvested and weighed (Table 3; Fig. S2). The weight of the lungs was used as an indication of the local inflammatory response. The mean weight of the lungs from the positive control mice vaccinated with live EBs was  $0.24 \pm 0.01$  g vs.  $0.36 \pm 0.01$  g for the negative controls ( $P < 0.05$ ). The weights of the lungs from mice vaccinated with nMOMP/Z3-14 ( $0.24 \pm 0.01$  g), nMOMP/A8-35 ( $0.23 \pm 0.01$  g), rMOMP/Z3-14 ( $0.29 \pm 0.02$  g) and rMOMP/A8-35 ( $0.28 \pm 0.01$  g) were all significantly lower than those of the negative control group ( $0.36 \pm 0.01$  g;  $P < 0.05$ ). Significant differences were also found in the weight of the lungs from the mice



**Fig. 7.** Number of *C. trachomatis* MoPn IFU recovered from the lungs at day 10 following i.n. challenge. Dots represent individual animals and the horizontal bars correspond to the medians for the different groups.

immunized with nMOMP compared with those immunized with rMOMP ( $P < 0.05$ ).

### 3.8. Recovery of *C. trachomatis* from the lungs

The lungs were homogenized and the number of chlamydial IFUs determined using monolayers of HeLa cells (Fig. 7; Table 3). In the positive control group immunized i.n. with live EBs the median number of IFU was below 50, the limit of detection (range: <50–350). Twelve of the 16 animals in this group had IFU counts below the limit of detection. For the negative controls groups inoculated with MEM-0 the median IFU count was  $4,718,525 \times 10^3$  (range:  $681,450–23,460,000 \times 10^3$ ). A significant reduction in the number of IFUs recovered from the lungs was observed in the four groups of mice vaccinated with MOMP preparations when compared with the negative control inoculated with MEM-0 ( $P < 0.05$ ). However, the animals immunized with nMOMP had significantly lower IFU counts than those vaccinated with rMOMP ( $P < 0.05$ ). The most significant reduction was observed in

the mice immunized with nMOMP/A8-35 (median number of IFUs, 200; range:  $<50\text{--}3932 \times 10^3$ ). Five of the 16 mice in this group had IFU counts below the limit of detection. For the group vaccinated with nMOMP/Z3-14, the median was  $140 \times 10^3$  and the range was  $5\text{--}2333 \times 10^3$  which is significantly higher than for the nMOMP/A8-35 group ( $P < 0.05$ ). On the contrary, no statistically significant difference was found between the groups immunized with rMOMP/Z3-14 vs. rMOMP/A8-35 ( $P > 0.05$ ).

#### 4. Discussion

In this study, we have shown that vaccines utilizing recombinant and native preparations of the *Chlamydia* MOMP, formulated either with a detergent (Z3-14) or with an APol (A8-35), can elicit in mice a strong protective immune response against an intranasal challenge with *Chlamydia*. The protection obtained with nMOMP/A8-35 was more robust than that achieved with nMOMP/Z3-14. On the other hand, no difference in protection was observed between the rMOMP preparations formulated with either Z3-14 or A8-35. This suggests that the enhanced protection elicited by nMOMP/A8-35 results either from, a better preservation of the native structure of the nMOMP, or from a more efficient presentation to the immune system, rather than from an adjuvant effect of A8-35.

Most current vaccines are formulated using live or inactivated whole pathogens [39,40]. These vaccines possess intrinsic adjuvant activity as they contain pathogen associated molecules, such as LPS, and nucleic acids with the ability to activate Toll-like receptors (TLR) and other pattern recognition receptors in the host cells [41–43]. This leads to induction of a coordinated set of innate and adaptive immune responses. In addition, vaccines containing either live or inactivated whole organisms stand a good chance of maintaining the native structure of their antigenic components. Therefore, upon exposure to the pathogen, the immune system of the vaccinated individual is ready to elicit a highly specific and well-directed response. The recent use of highly purified antigens, such as synthetic peptides or recombinant proteins, which, for the most part, lack inherent adjuvant activity, has resulted in the need to develop new and safe immunomodulatory adjuvants to enhance and direct antigen-specific humoral as well as cellular immunity [44–46]. Furthermore, the chemical synthesis or expression of recombinant immunogens, poses structural challenges. Not only must the primary structure of the antigen be correct, but also, in many instances, the secondary, tertiary and quaternary structures need to be native-like since they are critical for inducing protection [47,48].

The MoPn nMOMP and its recombinant version, rMOMP, have the same amino acid sequence but their post-translational modifications are not characterized. Spectrophotometric analyses of MoPn nMOMP have shown that it is neither phosphorylated nor N-linked glycosylated [30]. The most obvious and likely the most immunologically significant difference between nMOMP and rMOMP is their secondary, tertiary and quaternary structures. While nMOMP is an SDS-resistant homotrimer with a  $\beta$ -barrel structure, the secondary and tertiary structures of rMOMP appear for the most part disordered. Upon SDS-PAGE, particularly without a reducing agent, rMOMP appears largely aggregated likely due to the presence of eight cysteines [19]. Its quaternary structure, if any, has not been characterized either but it definitely does not form the native-like SDS-resistant homotrimer. These structural differences most likely account for the lower degree of protection conferred by rMOMP vs. nMOMP-based vaccines [28].

Why vaccines formulated with nMOMP and APols are more effective than those comprising nMOMP and detergent can have several origins. In our vaccine formulation amphipols are playing two critical roles. One is to maintain the MOMP in solution, and therefore avoid the use of a detergent. In addition, amphipols

stabilize the MOMP and thus, eliminate the need for glutaraldehyde fixation. The fact that A8-35 does not improve the protection afforded by rMOMP argues against a general adjuvant effect. On the other hand, membrane proteins interact with APols in ways that, in many aspects, differ from interactions with detergents. It is known that APols generally stabilize the native structure of membrane proteins [24,25]. Here we have shown that A8-35 stabilizes the nMOMP. Therefore, one could possibly argue that the protein presented to the immune system may more closely resemble the native structure when nMOMP is trapped with A8-35 than when it is solubilized in Z3-14 and fixed with glutaraldehyde. Likely, crosslinking by glutaraldehyde is beneficial in the presence of detergent, despite the fact that it may damage some critical epitopes, because it stabilizes the structure of MOMP, while in the presence of amphipols stabilization by fixation offers little benefit and the balance of effects is inverted.

Another possibility is that the difference in protection is related to the fate of nMOMP following inoculation. Protein-bound detergent, indeed, is in a constant and rapid equilibrium with the solution. When membrane proteins in detergent solution are diluted under the CMC of the detergent, as happens upon injection, the proteins aggregate, and some degree of denaturation may result. Membrane protein-APol complexes, on the other hand, are extremely stable and do not dissociate even under extreme dilutions preventing protein aggregation [49,50]. This could make for a more efficient delivery of nMOMP to target cells.

Several immunological mechanisms could account for the differences in the levels of protection afforded by nMOMP/Z3-14 vs. nMOMP/A8-35 preparations.  $CD4^+$  Th1 cells appear to be necessary for resolving a chlamydial infection, whereas the role of  $CD8^+$  T cells remains controversial [51–53]. B cells and/or antibodies have also been found to play a part in controlling a chlamydial infection [54]. mAbs have been described that recognize linear and conformational epitopes in MOMP. Current evidence indicates that mAbs that recognize conformational epitopes, specifically the trimer of MOMP, are more effective at neutralizing, in vitro and in vivo, the infectivity of *Chlamydia* than mAbs directed at linear epitopes [55,56]. Miller et al. [57] reported similar findings with a recombinant F1 (rF1) antigen of *Yersinia pestis*. Mice immunized with monomeric rF1 were significantly less protected against a challenge than those vaccinated with multimeric rF1. The authors proposed that antibodies generated against the monomer may have lower affinity than those elicited by the multimeric form of rF1. Antibodies to conformational epitopes are also thought to be critical for the protective immune response elicited by recombinant vaccines against hepatitis B virus and human papillomaviruses [47,48].

In addition to the modulation of the antibody response it is also likely that the cellular immune responses are differently affected by nMOMP/A8-35 and nMOMP/Z3-14. Several groups have now established that the conformation of the antigen influences the T cell response. For example, Musson et al. [58] characterized the mechanisms of antigen presentation by  $CD4^+$  T cell epitopes of the *Y. pestis* Caf1 antigen. They showed that the degree of processing was dependent on the localization of the epitopes. Epitopes located in the globular domain of the protein were presented by newly synthesized MHC class II after low pH-dependent lysosomal processing. On the contrary, epitopes from a flexible strand of the protein were presented by mature MHC class II, independent of low pH, and did not require proteolytic processing. Also, Hanada et al. [59] found that cytotoxic T lymphocytes, isolated from a human with renal cancer, recognized human leukocyte antigen-A3 MHC class I molecules presenting a non-contiguous peptide generated by protein splicing. They [59] proposed that the peptide was produced by post-translational excision in the cytosol followed by ligation. Warren et al. [60] have also described an antigenic peptide recognized by  $CD8^+$  T lymphocytes consisting of two non-continuous

peptide segments spliced in reverse order to that in the native protein. The authors postulated that the cutting and splicing of the peptide most likely occurs within the proteasome by transpeptidation. APols have been shown to deliver membrane proteins or transmembrane peptides to preformed membranes, be they lipid vesicles, black lipid films, or the plasma membrane of cells [25]. It is conceivable that, in so doing, they modulate the way the antigen is processed as compared to delivery from a detergent solution. This may be particularly important for cysteine-rich proteins such as MOMP since the gamma-interferon-inducible lysosomal thioesterase (GILT) promotes major histocompatibility complex (MHC) class I- and II-restricted presentation and processing of antigens containing disulfide bonds [61,62].

In conclusion, we have shown for the first time that a vaccine formulated with APols and nMOMP, an integral membrane protein, induces a more robust protective immune response than the same antigen formulated with a detergent. This enhancement of the protective ability of nMOMP most likely results from a better preservation of its native structure by the APol and/or from a more efficient presentation of the antigen to cells in the immune system. Several subunit vaccines currently include integral membrane proteins [47,48]. The possibility that the protective ability of these vaccines can be enhanced by the use of APols should be explored.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2011.04.065.

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