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Vaccination with major outer membrane protein proteosomes elicits protection in mice against a *Chlamydia* respiratory challenge

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Abstract

Vaccines formulated with the *Chlamydia muridarum* native major outer membrane protein (nMOMP) have so far been shown to elicit the most robust protection against this pathogen. nMOMP is a membrane protein and therefore, detergents are used to keep it in solution. Detergents however, have toxic effects. To address this limitation, we tested a nMOMP proteosome vaccine and compared it for its ability to elicit protection against nMOMP solubilized in the detergent Z3-14. The two preparations were formulated with or without CpG + Montanide (C/M). As a control antigen we used ovalbumin. Mice vaccinated with nMOMP developed strong humoral and cell mediated *Chlamydia*-specific immune responses. Based on the IgG2a/IgG1 levels in serum and amounts of IFN-γ in splenocytes supernatants the immune responses were predominantly Th1-biased. The animals were subsequently challenged intranasally with 2×10^3 *Chlamydia* inclusion forming units (IFU) and the course of the infection was followed for 10 days when the mice were euthanized. Based on changes in body weight, weight of the lungs and number of IFU recovered from the lungs, mice immunized with nMOMP-Ps and nMOMP +Z3-14 adjuvanted with C/M showed the most robust protection. In summary, nMOMP-Ps should be considered as *Chlamydia* vaccine candidates.

Keywords

Chlamydia; vaccine; proteosomes; major outer membrane protein; detergents; mouse model

1. INTRODUCTION

Chlamydiae are ubiquitous pathogens in nature producing genital, respiratory, gastrointestinal and ocular infections [1-4]. Attempts to produce vaccines against *Chlamydia* were initiated in the 1960's [3, 5, 6]. Using whole inactivated and viable organisms, trials were performed in humans and in non-human primates [3, 6] . Some of these vaccine

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protocols induced protection. The protection however, was short-lived, and appeared to be serovar specific [3, 6, 7]. Furthermore, some of the immunized individuals developed a hypersensitivity reaction after re-exposure to *Chlamydia* [3, 6]. Although the exact cause of the hypersensitivity reaction has not been elucidated, it is attributed to an antigenic component present in *Chlamydia* [8]. Therefore, there is a need to formulate a subunit vaccine against *Chlamydia* [1, 9, 10].

The *C. muridarum* MOMP belongs to a family of proteins found in the outer membrane of Gram-negative bacteria that have a molecular mass of approximately 40 kDa and function as porins [11, 12]. A subunit vaccine using the native MOMP (nMOMP) trimer of *Chlamydia* was shown to induce protection equivalent to that elicited with live organisms [13]. Integral membrane proteins, such as MOMP, are kept soluble in aqueous solutions using detergents. Detergents however are toxic to cells and do not stabilize the structure of proteins [14-17]. To circumvent such limitations we prepared nMOMP proteosomes (nMOMP-Ps). Proteosomes are protein micelles, free of detergents that do not have toxic effects. Proteosomes from *Neisseria*, *Salmonella, Fusobacterium* and *Plasmodium* have been used as antigens and adjuvants to elicit protection in humans and animal models [18-22]. Here, to assess the feasibility of formulating a vaccine using *Chlamydia* proteosomes, we extracted the nMOMP trimer and prepared nMOMP-Ps.

2. MATERIAL AND METHODS

2.1. Growth of C. muridarum stocks

The *C. muridarum* [*Cm*; previously called *C. trachomatis* mouse pneumonitis (MoPn), strain Nigg II; ATCC VR 123] was purchased from the American Type Culture Collection (Manassas, VA, USA). Stocks of *Chlamydia* elementary bodies (*Cm*-EB) were prepared as previously described [23].

2.2. Preparation of C. muridarum nMOMP proteosomes

The extraction and purification of the native *C. muridarum* nMOMP has already been described [12]. Briefly, EB were washed twice with 10 mM PBS pH 7.4. The pellet was resuspended in 0.02 M Tris pH 7.4, 1.0 M NaCl, 0.012 M $MgCl_2$ and 1 mM phenylmethylsulfonyl fluoride (PMSF; Calbiochem, La Jolla, CA), sonicated twice for 30 seconds to resuspend the EB and incubated with 25 μg of DNase/ml for 2 h on ice with constant mixing. Following centrifugation the pellet was resuspended in 0.2 M phosphate buffer pH 5.5, containing 0.001 M each of EDTA and PMSF. [3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate] (CHAPS; Anatrace; Maumee, OH) and dithiothreitol (DTT; Roche Applied Sciences; Indianapolis, IN) were added to a final concentration of 2% and 0.1 M respectively and extracted for 2 h at 37°C. The pellet was extracted a second time with CHAPS and then with 2% [n-Tetradecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate] (Z3-14; Anatrace). After 2 h of incubation at 37°C with constant mixing the sample was centrifuged and the MOMP recovered in the supernatant.

The nMOMP was further purified using a 1 × 35 cm hydroxylapatite column, with the following modifications [23]. The column was equilibrated with 0.02 M phosphate buffer pH 5.5, containing 0.1% Z3-14 and 0.001 M each of EDTA, PMSF and DTT. The column was eluted with a linear gradient of phosphate buffer from 0.02 M to 0.5 M containing 0.1% Z3-14 and EDTA, PMSF and DTT as indicated above. The purity of the nMOMP preparation was assessed by, gel electrophoresis and amino acid sequencing. Purified nMOMP, identified by electrophoresis on 10% tricine SDS-PAGE and Western blot using the monoclonal antibody MoPn-40 to MOMP, was precipitated by centrifugation following the addition of 80% (v/v) ethanol and incubation overnight at −20°C. The protein was

resuspended in 10 mM HEPES, pH 7.2, with 10% D-octylglucoside (DOG; Anatrace), followed by extensive dialysis in PBS containing 0.02% NaN_3 for formation of detergent-free protein micelles (proteosomes) [24].

2.3. Immunization and challenge of mice

Three-week-old female BALB/c (H-2^d) mice were purchased from Charles River Laboratories (Wilmington, MA) and were housed at the University of California, Irvine, Vivarium. The UCI, Animal Care and Use Committee approved the animal protocols. Animals were immunized by the intramuscular (i.m.) and subcutaneous (s.c.) routes with nMOMP+Z3-14, or nMOMP-Ps, (10 μg/mouse/immunization) in combination with CpG-1826 [(C); TriLink Biotechnologies; 10 μg/dose/mouse/immunization] plus Montanide ISA 720 VG [(M)' SEPPIC Inc., Coley Pharmaceutical Group, Ontario, Canada] at a 70/30 (v/v) [13, 25]. As negative control, mice were immunized with ovalbumin (OVA; Sigma, St. Louis, MO) and the same adjuvants. Three other groups of mice were immunized by the same routes and with the same antigens nMOMP+Z3-14, nMOMP-Ps and OVA, respectively but without the adjuvants. The mice were boosted twice at 2-week intervals with the same vaccine preparations. Positive control mice were immunized by the intranasal (i.n.) route once with 2×10^3 inclusion forming units (IFU) of *C. muridarum* . A negative control group was inoculated i.n. with minimal essential medium (MEM-0). Mice were challenged i.n. with 2×10^3 IFU of the *C. muridarum* at four weeks after the last boost. Eight to ten mice were employed for each group and the experiment was repeated once.

2.4. Characterization of the humoral and cell mediated immune responses

To measure *Chlamydia*-specific antibodies, blood was collected from each mouse the day before the challenge. ELISA and Western blots were performed as previously described with *Cm*-EB as the antigen [13, 25]. To assess T cell memory responses an *in vitro* lymphoproliferative assay (LPA) was performed before the i.n. challenge as previously described [13]. Levels of IFN-γ in supernatants from splenic T cells stimulated with UV-inactivated EB for 48 hrs were determined using ELISA kits (BD Pharmingen, San Diego, CA [25]. The LPA was also used to assess the toxicity of the nMOMP+Z3-14 and nMOMP-Ps preparations using Concanavalin A (Con A) as a non-specific stimulant.

2.5. Evaluation of the infection following the i.n. challenge

After the i.n. challenge, mice were weighed for 10 days [25, 26]. At day 10 post-challenge (p.c.) mice were euthanized, their lungs weighed, homogenized and serial 10-fold dilutions were used to infect Hela-229 cells. Following centrifugation the plates were incubated for 30 hrs at 37°C in a 5% CO_2 incubator. Inclusions were visualized with *Chlamydia*-specific monoclonal antibodies and were counted [27].

2.6. Statistical analyses

The Mann-Whitney U test was used to compare the numbers of *Chlamydia* IFU and the Student *t*-test was performed to compare $CD4^+$ T-cell proliferative response, lung weight and body weight changes of mice. Repeated measures ANOVA analyses were conducted to compare changes in mean body weight.

3. RESULTS

3.1. Antibody response

The day before the i.n. challenge, serum samples were collected and *Chlamydia*-specific antibody titers were determined by ELISA using *Cm*-EB as the antigen. As shown in Figure 1, mice immunized with nMOMP+Z3-14+C/M and with nMOMP-Ps+C/M developed high

total IgG geometric mean titers (GMT; 114,940 and 51,200, respectively). These titers were significantly higher than those of the groups immunized with nMOMP+Z3-14 or nMOMP-Ps (4,031 and 5,702, respectively; $p<0.05$). The antibody titer in the positive control group inoculated i.n. with live EB was 32,254.

The IgG2a/IgG1 ratios, used as an indicator of the Th1/Th2 response, were 1.58 and 0.79 in nMOMP+Z3-14+C/M and the nMOMP-Ps+C/M vaccinated groups, respectively, supporting a balanced response. In the mice immunized with nMOMP+Z3-14, or with nMOMP-Ps, the ratios were lower (0.04 and 0.22, respectively) indicative of a Th2-biased response. The highest IgG2a/IgG1 ratio (2.51) was obtained in the group inoculated i.n. with *Cm*-EB.

The highest neutralization titers (GMT; range) were observed with sera from animals vaccinated using adjuvants in particular those immunized with nMOMP-Ps+C/M (1,417; 250-6,250). However, this was not significantly higher than the titer of mice vaccinated with nMOMP+Z3-14+C/M (750; 250-1,250; $p>0.05$). The positive control group, immunized with *Cm*-EB, had GMT of 750 (range of 50-1,250). Sera from mice immunized with OVA or MEM-0 were used as negative controls.

By Western blot, using *Cm*-EB as the antigen, mice immunized using the nMOMP and nMOMP-Ps had antibodies only to this protein (data not shown). The control group inoculated i.n. with *Cm*-EB mounted an antibody response to several proteins with MW higher than 100-kDa, to the 60-kDa-crp, the 60-kDa-hsp, MOMP, and the 28 kDa protein, as well as LPS. Sera from mice immunized with OVA or MEM-0, as well as the pre-immune sera, showed no immune-reactive bands by Western blot.

3.2. Cell mediated immune responses

To ascertain the levels of the cell-mediated immune responses elicited following immunization, splenic T-cells were collected from individual mice the day before the i.n. challenge and their ability to proliferate in the presence of *Cm*-EB was determined. As shown in Figure 2A and 2B, significant proliferative responses to *Cm*-EB were obtained in the groups of mice immunized with nMOMP-containing preparations when compared with control mice immunized with OVA and MEM-0 alone ($p<0.05$). Interestingly, no significant differences in the proliferative responses were observed among the nMOMP-immunized groups irrespective of whether or not CpG+Montanide were used. As expected, the most robust proliferative response was observed in the positive control mice vaccinated i.n. with live *Cm*-EB.

To determine the strength of the Th1/Th2 response the levels of IFN-γ and IL-4 were measured in the supernatants from splenocytes stimulated with *Cm*-EB (Figure 2C). Overall, animals immunized with nMOMP-Ps had higher IFN-γ levels than those vaccinated with the nMOMP+Z3-14. The highest IFN-γ levels were measured in animals vaccinated with nMOMP-Ps+C/M (40.03 ng/ml) followed by those immunized with nMOMP-Ps (28.90 ng/ml). The control mice inoculated i.n. with *Cm*-EB had very high levels of IFN-γ (132.75 ng/ml) while the negative controls immunized with OVA or MEM-0 had very low levels of IFN-γ. IL-4 levels in the supernatants of the splenocytes stimulated with EB were below the level of detection in all the groups (data not shown).

3.3. Toxic effects of the nMOMP-Ps and nMOMP+Z3-14 preparations

To assess the potential cell-toxicity of the nMOMP-Ps and nMOMP+Z3-14 preparations, splenic T-cells from naïve mice were stimulated non-specifically in vitro with Con-A. As shown in Figure 3, while T-cells stimulated with Con-A in the presence of nMOMP-Ps were

able to proliferative, cells stimulated in presence of nMOMP+Z3-14 were not, indicative of the toxicity of the detergent.

3.4. Changes in body weight following the intranasal challenge

Four weeks after the last immunization mice were challenged i.n. with 2×10^3 IFU of *C. muridarum*. As shown in Fig. 4, all groups had lost 2-5% of their initial body weight by day 3 p.c. Mice immunized with nMOMP+Z3-14+C/M and with nMOMP-Ps+C/M lost additional weight by day 4 p.c. and then maintained their body weight until day 10 p.c. Animals immunized with nMOMP+Z3-14 and with nMOMP-Ps lost weight up to day 8 p.c. and then maintained their body weight. By repeated measures ANOVA, all four groups of animals vaccinated with nMOMP formulations had lost significantly less body weight than their respective control groups immunized with OVA ($p<0.05$). Significant difference in body weight loss was also observed in mice vaccinated with nMOMP-Ps using adjuvants versus thoseonlu immunized with nMOMP or MEM-0 ($p<0.05$). By day 10 p.c. the positive control group inoculated i.n. with *Cm*-EB had gained 0.63% of their initial body weight while the negative control inoculated i.n. with MEM-0 had lost 12.3% of their body weight at day 0 ($p<0.05$) (Fig. 5A and Table 1). Of the four experimental groups the one that had lost less weight at 10 days p.c. was that vaccinated with nMOMP+Z3-14+C/M (−1.52%) while the animals that had lost most weight were those immunized with nMOMP-Ps (−4.94%). Significant differences in body weight loss were also observed between the groups of mice vaccinated with nMOMP+Z3-14, with or without adjuvants, while no differences were found between the mice vaccinated with nMOMP-Ps with or without adjuvants.

3.5. Lung weight

Following euthanasia the lungs were weighted. In comparison with their respective OVA-immunized groups, the animals that received the four preparations of nMOMP, as determined by the weight of their lungs, had significantly less local inflammatory response ($p<0.05$) (Fig. 5B and Table 1). The lung weights of nMOMP+Z3-14+C/M (0.17), nMOMP-Ps+C/M (0.18) and nMOMP-Ps (0.19) immunized animals were not significantly different from those of the positive control group inoculated i.n. with *Cm*-EB (0.17; $p>0.05$). The two groups that had lighter lungs were the mice vaccinated using nMOMP plus the adjuvants, in particular the nMOMP+Z3-14+C/M immunized mice (0.17). However, they were not significantly different from the nMOMP-Ps immunized groups ($p>0.05$). The animals that had heavier lungs were those inoculated i.n. with MEM-0 (0.26).

3.6. Burden of C. muridarum infection in the lungs

Ten days after the i.n. challenge the mice were euthanized and their lungs cultured (Fig. 5C and Table 1). The number of IFU in the lungs of mice immunized with the four nMOMP-containing formulations was significant lower than in mice immunized with OVA ($p<0.05$). For example, the median number of IFU recovered from the lungs of mice vaccinated with nMOMP+Z3-14+C/M was 11.6 (range: BLD – 242.3) × 10^3 IFU, that from mice vaccinated with nMOMP-Ps+C/M was 64.7 (range: 3.7 – 377.4) × 10^3 IFU while the group immunized with OVA+C/M was 37,370.0 (range: 35.1 – 2,201,500) × 10^3 IFU ($p<0.05$). Similarly, the median number of IFU recovered from the lungs of mice immunized with nMOMP+Z3-14 was 333.0 (range: 9.2 – 3,293) × 10^3 IFU and that from the mice vaccinated with the nMOMP-Ps was 38.1 (range: 0.05 – 4,033) × 10^3 IFU, while for the group immunized with OVA it was 191,475.0 (range: 312.6 – 3,108,000) × 10^3 IFU ($p<0.05$). The number of IFU recovered from the lungs of mice immunized with nMOMP-Z3-14 preparations was significantly different between the two groups while it was not significantly different when the two groups immunized with nMOMP-Ps were compared. The median number of IFU

from the lungs of mice inoculated with live *Cm*-EB was below the limit of detection (range: BLD-BLD).

4. DISCUSSION

In this work we show that vaccination of BALB/c mice with *C. muridarum* purified MOMP, formed in proteosomes (nMOMP-Ps), elicits strong antibody levels and proliferative T-cell responses. Furthermore, animals that were vaccinated with nMOMP-Ps showed significant protection following a respiratory chlamydial challenge as determined by changes in body weight, lung weight and number of *Chlamydia* IFU recovered from their lungs. To our knowledge this is the first time that a vaccine formulated with MOMP proteosomes has been shown to be protective against a chlamydial infection.

Currently, the most efficacious subunit vaccine to protect against *Chlamydia* is formulated using nMOMP [13, 25, 28] [29]. This vaccine has been shown to protect mice against genital and respiratory challenges and monkeys against an ocular infection [13, 25, 30]. This formulation however, requires the use of detergents to keep the MOMP in solution since this protein, like other intrinsic membrane proteins, is not soluble in aqueous solutions. Detergents do not stabilize the structure of the protein for long periods of time and therefore, alternative approaches are needed to formulate vaccines against pathogens [15]. In addition, as shown here, using an in vitro LPA assay, detergents have unwanted toxic effects [14, 16, 17]. In the case of porins from gram-negative bacteria, formulating them as Ps can be a very effective approach to use them in a most native-like form [18-20]. Here, to evaluate this alternative approach, we compared nMOMP-Ps and nMOMP+Z3-14 formulated with or without adjuvants.

Following vaccination with live *Chlamydia,* or nMOMP, protection of mice against a genital challenge is mainly dependent on $CD4^+$ Th1 cells, B cells and antibodies also play a critical role, while $CD8^+$ cells are not required [1, 28, 29, 31, 32]. Therefore, in this work, we choose to combine nMOMP with adjuvants that elicit both strong Th1 and B cell-dependent responses, namely the TLR9 agonist CpG-1826 and Montanide, a non-TLR-dependent adjuvant [33-36]. While immunization of mice with nMOMP+Z3-14 without adjuvants resulted in induction of a Th2-type response, vaccination using the nMOMP+Z3-14+C/M resulted in a Th1-biased immune response as shown by the serum IgG2a/IgG1 ratio. This is similar to the effect of immunization with live *Chlamydia* [29]. In contrast, vaccination with nMOMP-Ps, whether formulated with or without C/M as adjuvants, resulted in a fairly balanced Th1/Th2 response, as determined by the IgG2a/IgG1 ratio. Interestingly, independently of the adjuvants used, nMOMP-Ps induced higher levels of IFN-γ in T-cell culture supernatants from immunized animals, as compared to those immunized with MoPn +Z3-14, indicating that overall, nMOMP-Ps induced a stronger Th1 response. However, mice vaccinated with live *Cm*-EB had lower antibody titers but the most robust splenic cell proliferative response and the best protection, supporting the findings that CMI are the most important host response for a candidate vaccine [29, 37].

To determine the protective activity of these formulations, we choose the well-established mouse respiratory challenge model [25, 38-40]. The results of the three parameters that we used to determine protection, body and lung weights and number of IFU in the lungs, showed good correlation. To determine the systemic effect of the i.n. challenge, mice were weighted daily for 10 days. Following the intranasal challenge, mice vaccinated with nMOMP-containing preparations initially lost weight but then remained stable for the rest of the observation period. In contrast, the negative control groups immunized with OVA or MEM-0 continuously lost weight. Overall, the two groups vaccinated with nMOMP+Z3-14, or nMOMP-Ps and C/M as adjuvants, were better protected that the animals immunized

without adjuvants. This observation suggested that nMOMP proteosomes may have an intrinsic adjuvant effect but that this was not as efficient as that of more classical adjuvants. By day 4 p.c. the group vaccinated with *Cm*-EB had fully recovered their initial body weight, indicating that these animals were able to mount a very rapid protective immune response.

Next, to assess the control of *Chlamydia* infection at 10 days p.c., lung weight was used as an indicator of local inflammatory responses [25, 38]. Similar to what observed for the body weight, the two groups vaccinated with nMOMP-Ps and nMOMP+Z3-14 with C/M, showed a very significant level of protection. Mice vaccinated with nMOMP-Ps and nMOMP +Z3-14, without C/M as adjuvants, also showed protection when compared with control mice immunized with OVA.

When the number of *Chlamydia* IFU recovered from the lungs was examined, mice vaccinated with nMOMP-Ps and nMOMP+Z3-14 showed a very robust protection. When compared to control animals immunized with OVA, those two groups had approximately 4 to 5 log less IFU recovered from the lungs. Interestingly, in the group immunized with nMOMP-Ps alone, the median number of IFU was lower than that of the animals immunized with nMOMP-Ps+C/M. This may resemble the adjuvant effect previously shown for porin proteosomes from other gram-negative bacteria, such as *Neisseria* and *F. nucleatum* [18, 41]. This effect has been described to be TLR2-dependent. Recent work has shown that nMOMP-Ps induce TLR2-dependent cell activation *in vitro*, similar to the porins mentioned above [24]. The observed induction of immune responses as well as protective effect of nMOMP-Ps in mice immunized in the absence of additional adjuvants may be explained, at least in part, by such TLR2-dependent cell responses.

In conclusion, although these results in the mouse model cannot be directly extrapolated to humans, our observation of robust humoral and cell mediated immune responses induced following immunization of mice with *Chlamydia* nMOMP-Ps, suggests that such a proteosome-based vaccine formulation could be successful in inducing protection in humans. In addition, such vaccine may be formulated to contain antigens from another sexually transmitted bacterium, *Neisseria gonorrhoeae,* that has porins like nMOMP and it is often found as a co-infecting pathogen in humans with *Chlamydia* [42, 43]. A *Chlamydia* vaccine will have a tremendous impact on the epidemiology of these infections [44, 45].

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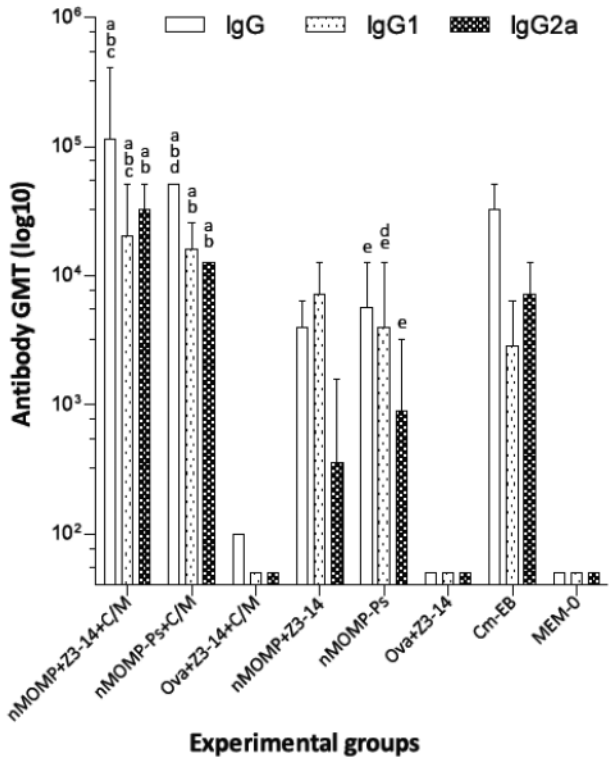

Figure 1. Serum antibody GMT (range) from the day before the i.n. challenge with *C. muridarum*

Mice were immunized with the *C. muridarum* nMOMP formulated in Z3-14, or as proteosomes, with/without the adjuvants CpG-1826 plus Montanide ISA 720 VG (C/M). As a negative control mice were immunized with ovalbumin instead of nMOMP. A positive control group was immunized i.n. with *C. muridarum* EB and a negative control with MEM-0.

[a], $p<0.05$ by Mann-Whitney Rank Sum Test compared to nMOMP+Z3-14 group
[b], $p<0.05$ by Mann-Whitney Rank Sum Test compared to nMOMP-Ps group
[c], $p>0.05$ by Mann-Whitney Rank Sum Test compared to nMOMP-Ps+C/M group
[d], $p>0.05$ by Mann-Whitney Rank Sum Test compared to *Cm*-EB group
[e], $p>0.05$ by Mann-Whitney Rank Sum Test compared to nMOMP+Z3-14 group

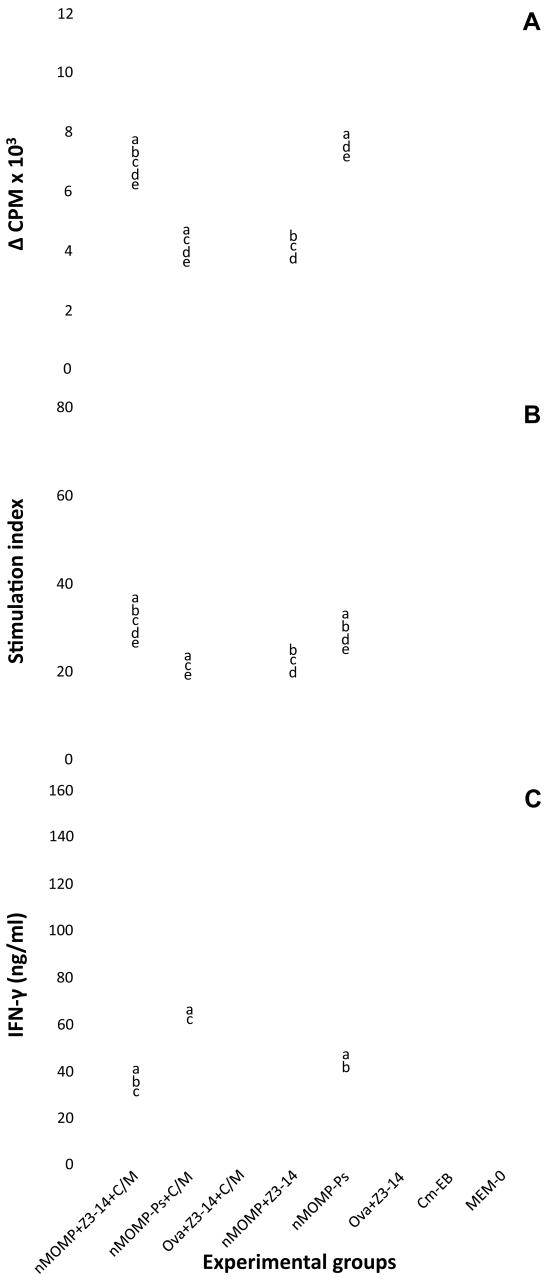

Figure 2. Cell mediated immune responses in vaccinated mice the day before the i.n. challenge with *C. muridarum*. A) T-cell proliferative responses as determined by the increase in counts per minute (CPM); B) T-cell proliferative responses: stimulation index (CPM from EB stimulated T-cells/CPM from medium stimulated T-cells. C) IFN-γ levels in supernatants from EB-stimulated T-cells

The ratio of UV-inactivated *Cm*-EB to antigen presenting cells was 1:1. The values are means ± 1 SD of four different cultures.

[a], $p<0.05$ by Mann-Whitney Rank Sum Test compared to OVA+Z3-14+C/M, OVA+Z3-14 or MEM-0 immunized groups.

[b], $p>0.05$ by Mann-Whitney Rank Sum Test compared to nMOMP-Ps+C/M immunized group.
[c], $p>0.05$ by Mann-Whitney Rank Sum Test compared to nMOMP-Ps immunized group.
[d], $p>0.05$ by Mann-Whitney Rank Sum Test compared to *Cm*-EB immunized group.
[e], $p>0.05$ by Mann-Whitney Rank Sum Test compared to nMOMP+Z3-14 immunized group.

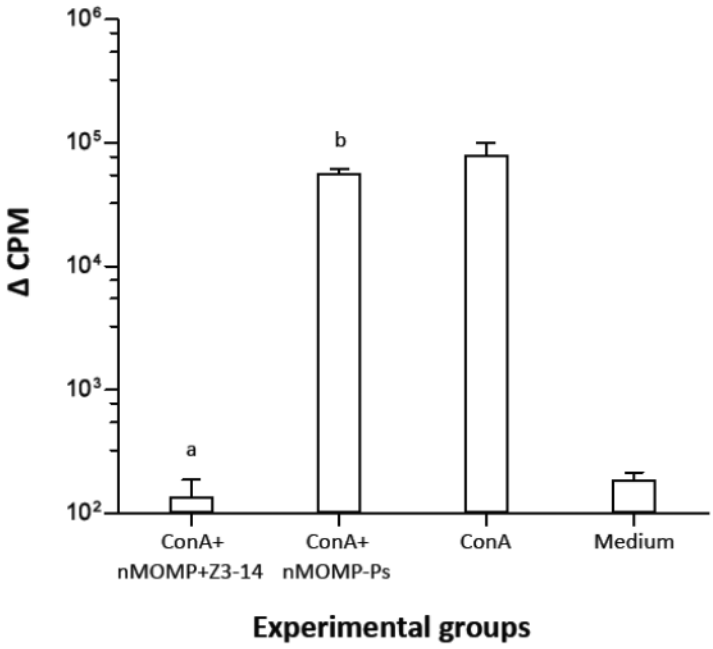

Fig. 3. T-cell proliferative responses in the presence of nMOMP+Z3-14 or nMOMP-Ps

To evaluate the possible toxic effects of nMOMP-Ps and nMOMP+Z3-14, T-cells from naive mice were stimulated in vitro with Con-A in the presence of the two nMOMP preparations. T-cell proliferative responses as determined by the increase in counts per minute (CPM). The values are means ± 1 SD of four different experiments.

[a], $p<0.05$ by Mann-Whitney Rank Sum Test compared to ConA or ConA+nMOMP-Ps stimulated groups and $p>0.05$ compared to Medium stimulated group.

[b], $p<0.05$ by Mann-Whitney Rank Sum Test compared to Medium stimulated group and $p>0.05$ compared to ConA stimulated group.

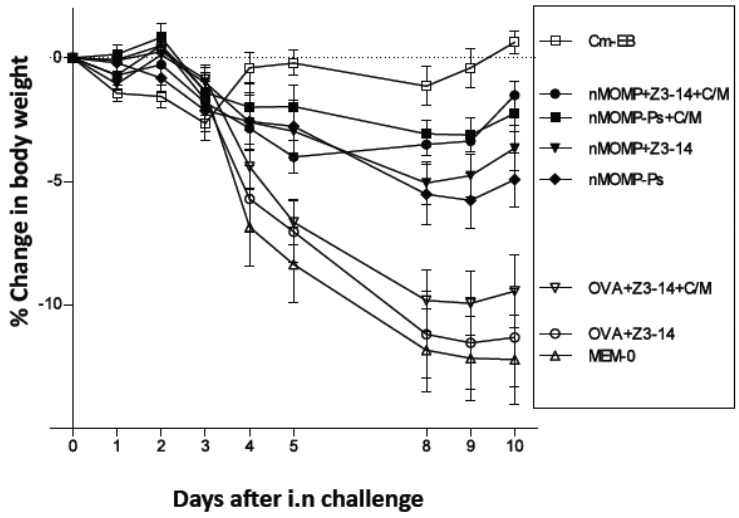

Figure 4. Changes in mean body weight of mice following the i.n. challenge with *C. muridarum*
Daily percentage change in mean body weight of each group of mice following the i.n. *C. muridarum* challenge.

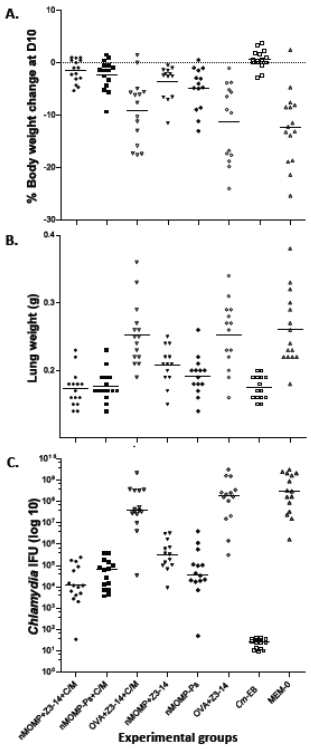

Figure 5. Percentage change in mice mean body weight, changes in lung weight and yields of *C. muridarum* IFU at day 10 p.c. A) Percentage change in body weight. The mean is indicated as a horizontal line; B) Changes in lung weight

The mean is indicated as a horizontal line; **C) *Chlamydia* yields.** The median number of IFU is shown as a horizontal line. Each symbol represents a single animal.

Table 1

Disease burden and yields of *C. muridarum* recovered from the lungs at day 10 p.c.

Experimental groups	% change in body weight mean ± 1SE	Lung weight mean ± 1SD	No IFU recovered from lungs X10^3 median (range)	
nMOMP+Z3−14+C/M	−1.52 ± 0.56 *a,b,f*	0.17 ± 0.03 *a,b,d,f,g*	11.6	(BLD-242.3) *h,i,j*
nMOMP-Ps+C/M	−2.25 ± 0.75 *a,c,d*	0.18 ± 0.02 *a,d,f,g*	64.7	(3.7 - 377.4) *i,j,k*
OVA+Z3−14+C/M	−9.14 ± 1.59	0.25 ± 0.05	37,370.0	(35.1 - 2,201,500.0)
nMOMP+Z3−14	−3.66 ± 0.89 *e*	0.21 ± 0.03 *e*	333.0	(9.2-3,293.0) *l*
nMOMP-Ps	−4.94 ± 1.08 *c,e*	0.19 ± 0.03 *c,e,g*	38.1	(0.05-4,033.0) *j,l*
OVA+Z3−14	−11.34 ± 1.98	0.25 ± 0.05	191,475.0	(312.6 - 3,108,000.0)
Cm-EB	0.63 ± 0.47	0.17 ± 0.02	BLD	(BLD-BLD)
MEM-O	−12.23 ± 1.80	0.26 ± 0.05	301,550.0	(1,665.0 - 3,089,500.0)

BLD: bellow the limit of detection

a, $p<0.05$ by the Student's *t*-test compared with the group immunized with OVA+Z3−14+C/M

b, $p>0.05$ by the Student's *t*-test compared with the group immunized with nMOMP-Ps+C/M

c, $p>0.05$ by the Student's *t*-test compared with the group immunized with nMOMP+Z3−14

d, $p>0.05$ by the Student's *t*-test compared with the group immunized with nMOMP-Ps

e, $p<0.05$ by the Student's *t*-test compared with the group immunized with OVA+Z3−14

f, $p<0.05$ by the Student's *t*-test compared with the group immunized with nMOMP+Z3−14

g, $p>0.05$ by the Student's *t*-test compared with the group immunized with *Cm*-EB

h, $p>0.05$ by the Mann Whitney's *U*-test compared with the groups immunized with nMOMP-Ps+C/M

i, $p<0.05$ by the Mann Whitney's *U*-test compared with the groups immunized with OVA+Z3-14+C/M

j, $p<0.05$ by the Mann Whitney's *U*-test compared with the groups immunized with nMOMP+Z3-14

k, $p>0.05$ by the Mann Whitney's *U*-test compared with the groups immunized with nMOMP-Ps

l, $p<0.05$ by the Mann Whitney's *U*-test compared with the groups immunized with OVA+Z3-14