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A TLR2 agonist is a more effective adjuvant for a *Chlamydia* major outer membrane protein vaccine than ligands to other TLR and NOD receptors

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Abstract

Chlamydia trachomatis (Ct) is the most common sexually transmitted bacterial pathogen in the World and there is an urgent need for a vaccine to prevent these infections. To determine what type of adjuvant can better enhance the immunogenicity of a *Chlamydia* vaccine, we formulated the recombinant major outer membrane protein (Ct-rMOMP) with several ligands for Toll-like receptor (TLR) and the nucleotide-binding oligomerization domain (NOD) including Pam₂CSK₄ (TLR2/TLR6), Poly (I:C) (TLR3), monophosphoryl lipid A (TLR4), flagellin (TLR5), imiquimod R837 (TLR7), imidazoquinoline R848 (TLR7/8), CpG-1826 (TLR9), M-Tri-DAP (NOD1/NOD2) and muramyl dipeptide (NOD2). Groups of female BALB/c mice were immunized intramuscularly (i.m.) three times with the Ct-rMOMP and each one of those adjuvants. Four weeks after the last immunization the mice were challenged intranasally (i.n.) with 10⁴ *C. trachomatis* mouse pneumonitis (MoPn) inclusion forming units (IFU). As negative antigen controls mice were immunized with the *Neisseria gonorrhoeae* recombinant porin B (Ng-rPorB) and the same adjuvants. As a positive vaccine control mice were inoculated i.n. with 10⁴ IFU of MoPn. The humoral and cell mediated immune responses were determined the day before the challenge. Following the challenge the mice were weighed daily and, at 10 days post-challenge (p.c.), they were euthanized, their lungs weighed and the number of IFU in the lungs counted. As determined by the IgG2a/IgG1 ratio in the sera, mice immunized with Ct-rMOMP + Pam₂CSK₄ showed a strong Th2 biased humoral immune response. Furthermore, these mice develop a robust cellular immune response with high *Chlamydia*-specific T cell proliferation and levels of IFN- γ production. In addition, based on changes in body weight, weight of the lungs and number of IFU recovered from the lungs, the mice immunized with Ct-rMOMP + Pam₂CSK₄, were better protected against the i.n. challenge than any group of mice immunized with Ct-rMOMP and the other adjuvants. In conclusion, Pam₂CSK₄ should be evaluated as a candidate adjuvant for a *C. trachomatis* vaccine.

Keywords

Chlamydia trachomatis; vaccine; mice; immunization; Toll-like receptors; Pam₂CSK₄

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INTRODUCTION

Pathogen-associated molecular patterns (PAMPs) are conserved structures of microbes recognized by the innate immune system [1]. PAMPs include microbial cell-wall components and nucleic acids or metabolic products including, lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acid, flagellin, CpG oligodeoxynucleotides motifs and ss- and ds-RNAs. Cells of the innate immune system recognize PAMPs by germ-line encoded pattern recognition receptors (PRRs). So far, four classes of PRRs have been identified: toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain (NOD) leucine rich-repeat containing receptors (NLRs), and retinoic acid-inducible gene I protein (RIG-I) helicase receptors [1]. TLRs were originally found by Lemaitre et al. [2] in fruit flies and since then, 13 mammalian TLRs have been discovered that are widely expressed on immune cells [3, 4]. Except for TLR3, the MyD88 dependent signaling pathway is the main gateway shared by most TLR members for triggering off innate and adaptive immunity [5, 6]. MyD88 deficient mice developed a profound defect in the antigen-specific Th1 but not Th2 response, suggesting that TLR signals play an influential role in the immune balance toward to Th1 but not Th2 response [6]. Therefore, there is an increase interest in using natural ligands, or synthetic agonists, for well-defined PRRs as adjuvants for subunit vaccines [2, 5, 6].

C. trachomatis is worldwide the leading cause of bacterial sexually transmitted diseases and can also produce gastrointestinal and respiratory infections [7, 8]. Annually, up to 4–5 million new genital *C. trachomatis* infections are reported in the United States [7, 9]. Although effective antimicrobial therapy is available, over 50% of the chlamydial infections are asymptomatic and even in symptomatic cases treatment failures can occur [10]. Furthermore, countries that have established screening programs followed by antibiotic therapy have observed an increase in the prevalence of the infection [11]. This increase is thought to be due to a block in the development of natural immunity as a result of the antibiotic therapy [11, 12]. Therefore, the development of an effective vaccine appears to be necessary if we want to control and eradicate these diseases [13–15]. A vaccination program will have a tremendous impact in the prevalence of these diseases [16].

By infecting female mice with live *C. trachomatis* MoPn it was determined that protection against a challenge required cellular and humoral immune responses [14, 17]. Specifically, CD4⁺ T cells, in particular Th1-type, and antibodies were found to be critical for the resolution of a genital chlamydial infection while CD8⁺ T cells played a minor role [14, 17–20]. For instance, transfer of purified CD4⁺ and CD8⁺ T cells from mice with a primary MoPn genital infection to naïve mice showed that only CD4⁺ cells conferred protection against a vaginal challenge [17–19]. Thus, combining cellular and humoral responses may be desirable for maximum immune-induced protection against *Chlamydia*.

So far CpG appear to be the most effective adjuvant for inducing a protective immune response in animals against a chlamydial challenge [21–24]. Here, to determine if other types of adjuvants can be more effective than CpG, we screened several TLRs and NODs agonist for their ability to adjuvantize a vaccine formulated with a *Chlamydia* rMOMP. Our data shows that, Pam₂CSK₄, a synthetic diacylated lipopeptide and a ligand for TLR2, is a very promising adjuvant for a *C. trachomatis* vaccine.

MATERIALS AND METHODS

C. trachomatis stocks

The *C. trachomatis* MoPn strain Nigg II (also called *Chlamydia muridarum*) was obtained from the American Type Culture Collection (ATCC; Manassas, VA) and was grown in

HeLa-229 cells using Eagles' minimal essential medium supplemented with 5% fetal bovine serum (FBS) [23]. Purified elementary bodies (EB) were stored at -70°C in 0.2 M sucrose, 20 mM sodium phosphate (pH 7.4), and 5 mM glutamic acid (SPG) [25]. Bacterial stocks were titrated in HeLa-229 cells.

Cloning of the *C. trachomatis* recombinant major outer membrane protein (Ct-rMOMP) and the *Neisseria gonorrhoeae* recombinant porin B (Ng-rPorB)

Genomic DNA from *C. trachomatis* MoPn strain Nigg II and *N. gonorrhoeae* strain FA 1090 (ATCC) were extracted using the Wizard genomic DNA Purification Kit (Promega, Madison, WI) [26]. The MoPn MOMP gene (GenBank, accession no. AE002272, X63409) was amplified without the leading sequence with *pfu* Turbo DNA Polymerase (Stratagene, La Jolla, CA) using the following primers; forward primer: 5' ACGCCCATGGCACTGCCTGTGGGGAATCCTGCT 3', and reverse primer: 5' AGCGGTCGACTTAGAAACGGAAGTGAAGCATT 3'. The Ng-rPorB (36 kDa) without the leading sequence (GenBank ID: AAW90430) was amplified by the PCR with the following primers: forward primer, Ngo-F2: 5' TATGCCATGGCCGATGTCACCCTG 3' and reverse primer, Ngo-R1: 5' GCGGATCCTTAGAATTTGTGGCGCAG 3'. The DNA products were cloned into pET-45b vector (Novagen, Gibbstown, NJ) and transformed into *Escherichia coli* TOP 10 competent cells as described [26]. After confirmation of positive clones by sequencing, the plasmid was transformed into *E. coli* BL21 (DE3) competent cells for expression by induction with 0.4 mM IPTG [26].

Antigens purification

The recombinant proteins were extracted from the *E. coli* inclusion bodies as described by Marston [27]. The pellet of the Ct-rMOMP was solubilized in TEN buffer with 8 M urea, 0.1 mM PMSF and 0.02 mM DTT to a concentration of 10 mg/ml. Following solubilization the MOMP was loaded onto a Sephacryl-S-300 column (1×50 cm; Sigma), which was pre-equilibrated with 100 mM Tris-HCl, pH 8.0, 200 mM NaCl, 10 mM EDTA, 0.2 mM DTT, and 0.05% Z3-14 and the peak fractions were pooled [23, 26, 28, 29]. The Ng-rPorB pellet was solubilized in 200 mM Tris, pH 8.2, 6 M Gdn-HCl, 2 mM EDTA, 1 mM PMSF and 20 mM DTT and centrifuged at $12,000 \times g$ for 20 min. Before immunization, both recombinant proteins were dialyzed against PBS (pH 7.4) with 0.05% Z3-14, and stored at -80°C [23].

The apparent MW and purity of Ct-rMOMP and Ng-rPorB were determined by 10% tricine-SDS-PAGE [30]. Using the limulus amoebocyte assay (BioWhittaker, Inc., Walkersville, MD), both recombinant proteins were found to have less than 0.05 EU of LPS/mg of protein.

Immunization protocols

Three-week-old female BALB/c (H-2^d) mice (Charles River Laboratories; Wilmington, MA) were housed at the University of California, Irvine, Vivarium. The University of California, Irvine, Animal Care and Use Committee approved all animal protocols.

Agonists for TLRs and NODs including, Pam₂CSK₄ (TRL2/6), Poly (I:C) (TLR3), monophosphoryl lipid A (MPL-A) from *Salmonella minnesota* (TLR4), flagellin from *Bacillus subtilis* (TLR5), imiquimod R837 (TLR7), imidazoquinoline R848 (TRL7/8), MurNAc-L-Ala- γ -D-Glu-mDAP (M-Tri-DAP) (NOD1/NOD2) and muramyl dipeptide (MDP) (NOD2) were obtained from InvivoGen (San Diego; CA). CpG-1826 (TLR9) was purchased from TriLink (San Diego, CA). These adjuvants were directly mixed with the rMOMP (10 $\mu\text{g}/\text{mouse}/\text{immunization}$) using previously published dosages (Tables 1 & 2) [23, 31–35].

For the initial pilot experiment five mice were vaccinated i.m. 3 times at 2-week intervals (Table 1) with each adjuvant plus Ct-rMOMP. Additional experiments were performed as shown in Table 2. Positive vaccine control mice were immunized i.n. once with 10^4 IFU of the *Chlamydia* MoPn in 20 μ l of modified Eagle's medium without serum (MEM-0; Invitrogen, Carlsbad, CA). As a negative antigen control, mice were immunized with the Ng-rPorB. A second negative control group was inoculated once i.n. with 20 μ l MEM-0 [22].

Characterization of the humoral response by enzyme-linked immunosorbent assay (ELISA) and Western blot assay

To measure *Chlamydia*-specific antibody titers following immunization, blood was collected from each mouse the day before the challenge. ELISA was performed as previously described [23]. Each well of a 96-well plate was coated with 100 μ l per well of MoPn EB containing 10 μ g/ml of protein in PBS to which 1-fold serial dilutions of serum were added. Following incubation at 37°C for 1 hr, the plates were washed, and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Ig), IgG1, IgG2a (BD Pharmingen, San Diego, CA), and IgG (mixed of IgG1, IgG2a, IgG2b and IgG3; Southern Biotechnology Associates, Birmingham, AL) and IgA (ICN Pharmaceuticals, Aurora, Ohio) were incubated at 37°C for 1 hr. The binding was measured in an ELISA reader (Labsystem Multiscan; Helsinki, Finland) at 405 nm using 2, 2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonate) as the substrate. The geometric mean titers (GMT) are expressed as the inverse of the highest dilution that gave a positive result.

The Western blot was performed as previously described with MoPn EB as the antigen [23]. Approximately 30 μ g of purified EB were loaded on a 7.5-cm-wide slab polyacrylamide gel. Following transfer to a nitrocellulose membrane, the nonspecific binding was blocked with BLOTTO (bovine lacto transfer technique optimizer; 5% [wt/vol] nonfat dry milk, 2mM CaCl_2 , 50 mM Tris-HCl [pH 8.0]) for 2 hrs at room temperature, and then serum samples diluted 1:100, were added to the membrane strips and incubated overnight at 4°C. The membrane was washed and incubated with horseradish peroxidase-conjugated goat anti-mouse antibody for 1 hr, followed by visualization of the bands by developing with 0.01% hydrogen peroxide and the substrate 4-chloro-1-naphthol.

In vitro splenic T cell response by lymphocyte proliferative assay (LPA) and cytokines assay

To assess the T cell memory response after the immunization, an *in vitro* LPA was performed before the i.n. challenge as previously described [22, 36]. In brief, spleens of four mice from each group were harvested and disrupted individually in PBS containing 2% FBS. Erythrocytes were lysed with ammonium chloride buffer (BD Pharm Lyse, BD Biosciences). T cell-enriched lymphocytes (10^5 cells/well) were isolated through a nylon wool column. The T cell-enriched lymphocytes were cultured with accessory cells for antigen presentation (APCs; 10^5 /well) which were prepared by irradiating (3,000 rads; ^{137}Cs) syngeneic unseparated splenocytes and incubated with UV-inactivated *C. trachomatis* MoPn EB at a 1:1 ratio, at 37°C for 2 hrs. Concanavalin A (ConA; Sigma-Aldrich) was used as a positive stimulant at a concentration of 5 μ g/ml, and tissue culture media served as a negative control. At the end of the 96 hrs of incubation, 1.0 μ Ci of [*methyl*- ^3H] thymidine (47 Ci/mmol; Amersham, Arlington Heights, IL) were added to each well and the uptake of the [^3H] thymidine was measured after 18 hrs using a scintillation counter (Beckman Instruments, Fullerton, CA).

Levels of IFN- γ , IL-10, and IL-4 in supernatants from splenic T cells stimulated for 24 hrs were determined using ELISA commercial kits (BD Pharmingen, San Diego, CA) as described [36].

Intranasal challenge

After the last immunization the animals were rested for 4 weeks and their body weight measured the day of the challenge. The animals were challenged i.n. with 10^4 IFU of the *C. trachomatis* MoPn under Xylazene/Ketamine anesthesia and for 10 consecutive days their body weight was monitored as previously described [37]. At day 10 post-challenge (p.c.) the mice were euthanized, their lungs were harvested, weighed, homogenized in 5 ml of SPG, and serial 10-fold dilutions were inoculated onto Hela-229 cells grown in 48-well tissue culture plates. Following centrifugation the plates were incubated for 30 hrs at 37°C in a 5% CO₂ incubator. The chlamydial inclusions were stained with a cocktail of mAb prepared in our laboratory and were counted [36]. The limit of detection per pair of lungs/mouse was 50 IFU.

Statistical analyses

Statistical analyses were performed with the SigmaStat software package on a McIntosh computer (Cupertino, CA.) The two-tailed unpaired Student's *t*-test, the Mann–Whitney *U*-test, and repeated measures ANOVA were employed to determine the significance of the differences between groups. Differences were considered significant for values of $p < 0.05$.

RESULTS

Adjuvant pilot study

To select adjuvants, that in combination with the Ct-rMOMP, induce a protective immune response in mice against an i.n. challenge, we performed a pilot experiment with nine different TLR and NOD agonists. As shown in Table 1, using the IgG2a/IgG1 ratio as an indicator of the Th1 versus Th2 response, CpG-1826 elicited a predominant Th1 response, Pam₂CSK₄, imidazoquinoline R848, M-Tri-DAP, muramyl dipeptide, imiquimod R837 and flagellin induced a biased Th2 response, while Poly (I:C) and MPLA elicited a balanced Th1 versus Th2 response.

The mice were weighed for 10 consecutive days after the challenge and following euthanasia, their lung were harvested, weighted and the number of IFU present in the lungs determined (Table 1). A decrease in body weight is an indication of the severity of the infection, and therefore the effectiveness of the vaccination to elicit a protective response, while the weight of the lung is an indication of the local inflammatory reaction. In comparison with the control inoculated with MEM-0, animals immunized with Ct-rMOMP + Pam₂CSK₄ or Ct-rMOMP + Poly (I:C) showed the best protection as determined by body weight change, lung weight, and *Chlamydia* loads while those vaccinated using Ct-rMOMP + MPLA or Ct-rMOMP + imidazoquinoline R848 showed a weak protection (Table 1). Based on these results we decided to do further testing to compare Pam₂CSK₄, Poly (I:C) and CpG-1826 since they elicited the strongest protection and, based on the IgG2a/IgG1 ratio, induced different immune responses.

Humoral immune response

To further characterize the protective response elicited by the three selected adjuvants in combination with rMOMP additional groups of mice were immunized with Ct-rMOMP + Pam₂CSK₄, Ct-rMOMP + CpG-1826 or Ct-rMOMP + Poly (I:C) (Table 2). In addition, three negative control groups were immunized with Ng-rPorB and the three adjuvants. Also,

a negative control group was inoculated i.n. with MEM-0 and a positive control was immunized i.n. with live MoPn EB.

Serum samples from all the groups of mice were collected the day before the i.n. challenge and tested for *Chlamydia*-specific antibodies by an ELISA (Table 3). Mice immunized with Ct-rMOMP + Pam₂CSK₄ had the highest IgG (64,508) and IgA (8,063) GMT in comparison to the mice immunized with Ct-rMOMP + Poly (I:C) (IgG= 32,254 and IgA= 1,600) or Ct-rMOMP + CpG-1826 (IgG=40,637 and IgA=800). The levels of IgA were significantly different between the mice vaccinated with Ct-rMOMP + Pam₂CSK₄ and the other two groups ($p < 0.05$). Consistent with the pilot experiment, based on the IgG2a/IgG1 ratio, Pam₂CSK₄ elicited a Th2 bias (IgG2a/IgG1=0.02), Poly (I:C) produced a balanced response (IgG2a/IgG1=1.6), whereas CpG-1826 (IgG2a/IgG1=6.4), induced an immune response with Th1 polarity. The positive control group immunized with live EB elicited high IgG (51,200) and IgA (4,032) levels and a Th1-based response (IgG2a/IgG1=25.4). No detectable IgG or IgA anti-*Chlamydia* antibodies were found in the mice immunized with Ng-rPorB, and the same three adjuvants, or MEM-0.

The Western blot shows that mice vaccinated with the Ct-rMOMP developed antibodies only against this protein, as indicated by a band with a molecular weight (MW) of 40 kDa (Fig. 1). Animals immunized i.n. with live *C. trachomatis* produced antibodies against components with a molecular mass higher than 100 kDa, the 60-kDa cysteine rich protein (crp), the 60-kDa-heat shock protein (hsp), MOMP, the 28 kDa protein and LPS. Control mice immunized with Ng-rPorB or MEM-0 showed no bands by Western blot.

Cell mediated immune response (CMI)

Cell-mediated immune responses elicited by immunization were evaluated by an in vitro LPA and cytokine assay using the splenic T-cells the day before the i.n. challenge (Table 4). The proliferative response of the splenic T-cells stimulated with EB in the mice immunized with Ct-rMOMP + Pam₂CSK₄ ($6,653 \pm 82$ cpm, SI=23) and Ct-rMOMP + Poly (I:C) ($5,101 \pm 71$ cpm, SI=25) were significantly stronger than the response of the mice immunized with Ct-rMOMP + CpG-1826 ($3,544 \pm 60$ cpm, SI=14, $p < 0.05$) or their respective controls immunized with Ng-rPorB or MEM-0 ($p < 0.05$). As expected the most robust lymphoproliferative response was observed in the mice immunized i.n. with live *C. trachomatis* ($7,503 \pm 87$ cpm, SI=45).

The level of IFN- γ produced by the T-cells of the mice immunized i.m. with Ct-rMOMP + Pam₂CSK₄ ($9,149 \pm 1,896$ pg/ml) was significantly higher than that from the mice immunized with Ct-rMOMP + Poly (I:C) ($1,532 \pm 240$ pg/ml, $p < 0.05$) or with Ct-rMOMP + CpG-1826 ($1,161 \pm 60$ pg/ml, $p < 0.05$) or the control groups immunized with Ng-rPorB or MEM-0 ($p < 0.05$) (Table 4). The highest level of IFN- γ was detected in the supernatant of activated T cells from mice vaccinated i.n. with live *C. trachomatis* ($23,639 \pm 2,506$ pg/ml). The levels of IL-10 were low and no differences were found between all the groups of mice. IL-4 was only detected in supernatants from mice immunized with Ct-rMOMP + Pam₂CSK₄ (18.6 ± 1.0 pg/ml) and those inoculated with live EB (22.6 ± 1.9 pg/ml).

Changes in body weight of the mice following the i.n. challenge

The daily changes in mean body weight of each group of mice following the i.n. challenge are shown in Fig. 2A. All mice, except those immunized i.n. with live EB, rapidly lost weight from days 2 to day 4 p.c. Afterward, the mice immunized with Ct-rMOMP + Pam₂CSK₄ regained their weight up to the end of observation at 10 days p.c. In contrast, the mice immunized with Ct-rMOMP + Poly (I:C) or Ct-rMOMP + CpG-1826 regained less body weight. The control mice vaccinated with Ng-rPorB + Pam₂CSK₄, Ng-rPorB + Poly

(I:C), Ng-rPorB + CpG1826 or with MEM-0 consistently lost weight during the 10 days of observation. The mice immunized with Ct-rMOMP + Pam₂CSK₄ showed significant less loss of body weight over the 10 days of observation in comparison with the mice immunized with Ct-rMOMP + Poly (I:C), Ct-rMOMP + CpG-1826, Ng-rPorB + Pam₂CSK₄, or with MEM-0 ($p < 0.001$ by repeated measured ANOVA). There was no significant difference in body weight between the mice immunized with Ct-rMOMP + Poly (I:C) and those vaccinated with Ct-rMOMP + CpG-1826 ($p > 0.05$ by repeated measured ANOVA).

As shown in Fig. 2B and Table 5, at day 10 p.c the mice immunized with Ct-rMOMP + Pam₂CSK₄ i.m. had lost $4.8 \pm 0.8\%$ of their initial mean body weight. This was significantly less than the mice immunized with Ct-rMOMP + Poly (I:C) or Ct-rMOMP + CpG-1826 or the negative controls inoculated with Ng-rPorB + Pam₂CSK₄ or MEM-0 ($11.3 \pm 1.2\%$; $11.0 \pm 1.2\%$; $13.7 \pm 1.7\%$ and $17.5 \pm 1.1\%$, respectively; $p < 0.05$). The positive control mice immunized i.n. with live EB maintained their weight and by day 10 p.c. was $+0.7 \pm 0.6\%$ above day 0.

Burden of *Chlamydia* infection in the lungs

Ten days after the i.n. challenge the mice were euthanized and their lungs were harvested, weighed, homogenized and cultured. As shown in Fig. 3A and Table 5, the mean weight of the lungs from the mice immunized with the Ct-rMOMP + Pam₂CSK₄ was 0.27 ± 0.01 g was significantly lighter than that from the control mice inoculated i.n. with MEM-0 (0.32 ± 0.01 g, $p < 0.05$). However, there was no difference in comparison to that of the mice immunized with Ct-rMOMP + Poly (I:C) (0.27 ± 0.01 g), or Ct-rMOMP + CpG-1826 (0.28 ± 0.01 g), or Ng-rPorB + Pam₂CSK₄ (0.26 ± 0.02 g; $p > 0.05$, respectively). The mean weight of the lungs of the positive control mice vaccinated i.n. with live EB was 0.20 ± 0.01 g, significantly different with all the other groups ($p < 0.05$).

The yields of chlamydial IFU recovered from the lungs are shown in Fig. 3B and Table 5. The median number of IFU recovered from both lungs of the mice vaccinated with the Ct-rMOMP + Pam₂CSK₄ was 6 ($0.04 - 314$; range) $\times 10^6$ IFU. This was significant less number of IFU than the mice immunized with Ct-rMOMP + Poly (I:C) [$26 (3.0 - 2,053) \times 10^6$ IFU, $p < 0.01$], or with Ct-rMOMP + CpG-1826 [$28 (0.009 - 4,421) \times 10^6$ IFU; $p < 0.01$], or Ng-rPorB + Pam₂CSK₄ [$117 (0.9 - 1,628) \times 10^6$ IFU, $p < 0.01$], or inoculated i.n. with MEM-0 [$264 (6 - 9,966) \times 10^6$ IFU, $p < 0.01$]. Consistent with the body weight changes, there is no statistical difference in the *Chlamydia* loads between the mice immunized with Ct-rMOMP + Poly (I:C) and Ct-rMOMP + CpG-1826 ($p > 0.05$). The median number of IFU from the lungs of mice vaccinated with live organisms was below the level of detection (< 50 IFU/lungs/mouse).

DISCUSSION

In this study we have shown that vaccines formulated with the *Chlamydia* rMOMP, in combination with Pam₂CSK₄, CpG-1826 and Poly (I:C) as adjuvants, elicited in mice significant humoral and cellular immune responses. Based on the IgG2a/IgG1 ratio these three adjuvants induced Th2, Th1 and mixed Th1/Th2 responses, respectively. Levels of IFN- γ were high in T-cell supernatants from animals vaccinated with Pam₂CSK₄, CpG-1826 and Poly (I:C). A robust protection against an i.n. challenge with *Chlamydia* was observed in the three groups of mice in comparison to the control animals immunized with Ng-rPorB rather than rMOMP. Particularly strong protection was observed in the mice immunized with rMOMP + Pam₂CSK₄. This group of mice lost significantly less body weight, during the 10 days following the i.n. challenge, than the mice immunized with rMOMP + CpG-1826 or rMOMP + Poly (I:C). In addition, the lungs of the animals vaccinated with rMOMP + Pam₂CSK₄ weighed less and yielded a significant lower number of *Chlamydia*

IFU when compared with the rMOMP + CpG-1826 or rMOMP + Poly (I:C) immunized groups.

Most current vaccines are formulated with live or inactivated whole pathogens [38, 39]. These vaccines possess intrinsic adjuvant activity as they contain PAMPs, such as LPS, and nucleic acids that activate PRRs in the host cells leading to a strong induction of a coordinated set of innate and adaptive immune responses [40–44]. Therefore, upon exposure to the pathogen, the immune system of the vaccinated individual is ready to mount a robust and highly specific response. The recent use of highly purified antigens, such as synthetic peptides or recombinant proteins, which, for the most part, lack inherent adjuvanticity, has resulted in the need to develop new adjuvants to enhance and direct antigen-specific humoral and cellular immune responses [35, 45–47]. Here, to test the most efficacious PAMPs to use as an adjuvant for a *Chlamydia* vaccine we performed a pilot experiment to test nine TLR and NOD agonists. Our results showed that the strongest immune response and the best protection were obtained when the *Chlamydia* rMOMP was adjuvanted with Pam₂CSK₄, a TLR2 agonist.

It is in general assumed that natural infection elicits the best protection against subsequent exposure to the same pathogen [43, 44]. Therefore, adjuvants used in vaccination should utilize the same signaling pathway that the pathogen is going to effect at the time of infection. *Chlamydia* infection has been shown to elicit TLR- and Nod1-mediated activation. Several chlamydial PAMPs appear to be involved in this process including LPS, macrophage infectivity potentiator (Mip), heat shock protein 60 (hsp60), peptidoglycan and plasmid-dependent factors [48–52]. Different PRRs have been described that recognize *Chlamydia* signaling in particular TLR4 and TLR2 [52–55]. TLR4 is the main receptor for LPS. The *Chlamydia* LPS however, is approximately 100-fold less potent in its endotoxin activity when compared with the LPS of other organisms such as *E. coli*, *Salmonella minnesota* and *N. gonorrhoeae* and therefore, its adjuvanticity is very limited [48, 52]. Mip is a lipoprotein that is conserved across chlamydial species and has been shown to be present at the surface of EB [56]. The proinflammatory response to Mip is strong and is mediated mainly by its lipid component through TLR2/TLR1/TLR6 with the help of CD14 [48, 56]. Furthermore, O'Connell et al. [50] have shown that TLR2 and MyD88 co-localize to the chlamydial inclusion and they proposed that TLR2 might be signaling from this intracellular location. Therefore, TLR2 maybe a critical PRR for *Chlamydia*.

Until recently it was thought that triacylated lipopeptides, such as Pam₃CSK₄, signaled through the TLR2-TLR1 heteromer, whereas diacylated lipopeptides, like the macrophage-activating lipopeptide 2-kDa from *Mycoplasma fermentans* (MALP-2) and Pam₂CSK₄ elicited signaling through TLR2-TLR6 heteromer [57–59]. Buwitt-Beckmann et al. [60, 61] using TLR1- and TLR6-deficient mice, have now shown that not only triacylated lipopeptides, but also diacylated lipopeptides, such as Pam₂CSK₄ and the elongated MALP-2 analog Pam2CGNNDENISFKEK-SK4 (MALP-2-SK4), induce B lymphocyte proliferation and TNF- α secretion in macrophage in a TLR1- and TLR6-independent manner. Therefore, for this group of lipopeptides, TLR-2 appears to be the main PRR. In murine macrophage cell lines, Pam₂CSK₄ elicits strong NF- κ B but relative low pro-inflammatory cytokines [59, 60]. In addition, Pam₂CSK₄ has been shown to induce a pattern of chemokines that is suggestive of the MyD88-independent TRIF-Mal-TIRAP pathway [57].

Differences in TLR expression and cell composition mean that TLR agonists produce different responses when administered by various routes [40, 62]. Here, we used the i.m. route for immunization and, as determined by the IgG2a/IgG1 ratio in serum, obtained a very strong Th2-biased response while a Th1 response was observed when considering the

levels of IFN- γ secreted in the EB-stimulated splenocytes. Like in our study, i.n. vaccination of BALB/c mice with recombinant HIV-1 p17 protein adjuvanted with synthetic MALP-2, as determined by the IgG2a/IgG1 ratio in serum, elicited a Th2-biased immune response [63]. However, based on the frequency of IFN- γ -secreting spleen cells, MALP-2 induced a Th1-biased cellular response [62]. These authors also showed that MALP-2 elicited high levels of HIV-1 p17-specific IgA in the lung and vaginal washes [62]. We also detected very high levels of *Chlamydia*-specific IgA in the mice vaccinated with rMOMP + Pam₂CSK₄. Also, Borsutzky et al. [63] showed that intradermal immunization of BALB/c with the HIV-1 Tat protein and MALP-2 elicited a Th2 response. Interestingly, animals that were subsequently boosted i.n. with the same vaccine modified the T helper pattern towards a dominant Th1 response. Therefore, in animal models lipopeptide, such as MALP-2 and Pam₂CSK₄, can mediate the immune responses towards a Th1 or a Th2 response. Strong humoral responses to this group of lipopeptides have also been observed using human cells. For example, Liang et al. [64] showed that Pam₂CSK₄ could induce CCR9 and CCR10 expression in human circulating B cells and increases chemotaxis to CCL25 and CCL28 two mucosal chemokines. Furthermore, these authors also demonstrated that human B cells stimulated with Pam₂CSK₄ increase J chain and IgA production and therefore, induction of mucosal-like antibody secreting cells.

Here, high levels of IgA and IFN- γ were elicited in mice immunized with rMOMP + Pam₂CSK₄. In humans, Brunham et al. [65] showed an inverse correlation between the titers of IgA in genital secretions and the quantity of *C. trachomatis* isolated from the cervix. In addition, by passively immunizing mice with an IgA monoclonal to MOMP, Pal et al. [66] showed strong protection against a *Chlamydia* genital challenge. IFN- γ is known to have in vitro and in vivo strong antichlamydial activity [17, 67, 68]. Therefore, the ability of Pam₂CSK₄ to induce high levels of IgA and IFN- γ , may be the underlying immune mechanism that results in strong protection when using this adjuvant. These results suggest that lipopeptides, such as Pam₂CSK₄, may be good adjuvants to formulate human vaccines for mucosal pathogens like *Chlamydia*.

TLR ligands induce a pro-inflammatory response and the challenge is to modulate them so that their positive action is significantly more pronounced than their toxicity [54, 69, 70]. To evaluate their toxic effects, it is critical to test these compounds in vivo. Based on our literature survey, it appears that Pam₂CSK₄ has not yet been used as a vaccine adjuvant but MALP-2 has. For example, Carzola et al. [71] immunized mice with cruzipain, a *Trypanosoma cruzi* antigen, formulated with MALP-2 and demonstrated strong humoral and cell mediated immune responses and protection against a trypanomastigote challenge. No significant toxic effects were observed in these experiments. More importantly, in a phase I/II clinical trial, patients treated with MALP-2 for inoperable pancreatic cancer, not only tolerated well this lipopeptide, but it prolonged their survival [58]. Therefore, it will appear that, at least some natural and synthetic lipopeptides, can safely be used in vaccine formulations. However we noticed that, while mice immunized with Ng-rPorB + CpG-1826 or Ng-rPorB + Poly (I:C) were not protected as compared to the animals inoculated with MEM-0, mice vaccinated with Ng-rPorB + Pam₂CSK₄ showed a low level of protection. Adjuvants by them own, elicit an immune response that is usually short-lived and non-specific [22, 34, 35]. Here, in order to minimize the non-specific immune response elicited by the adjuvants, after the last immunization, we waited for four weeks before we challenged the mice. In spite of waiting for four weeks, the negative control group of mice vaccinated with Ng-rPorB + Pam₂CSK₄ showed some protection, which suggested that we may need to wait for a longer period of time to challenge the mice after the last immunization or, we may need to decrease the dosage if we want avoid the non-specific protection elicited by this adjuvant.

In summary, the results presented here show that, the *Chlamydia* rMOMP adjuvanted with Pam₂CSK₄, a TLR2 agonist, elicits very strong cellular and humoral immune responses in mice. Importantly, the immune response conferred a robust protection against a respiratory challenge. Pam₂CSK₄ by itself, or in combination with other TLR agonists, may be a good adjuvant for chlamydial vaccines required to induce in humans strong antigen-specific systemic and mucosal cellular and humoral immune responses [13, 14, 17, 72].

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Figure 1. Western blot analysis of serum samples from the day before the i.n. challenge with *C. trachomatis*

C. trachomatis MoPn EB were used as the antigen. Lane 1, molecular weight standards. Lanes 2 to 7, sera from mice immunized with: Ct-rMOMP + Pam₂CSK₄; Ct-rMOMP + Poly (I:C); Ct-rMOMP + CpG-1826; Ng-rPorB + Pam₂CSK₄; Ng-rPorB + Poly (I:C); Ng-rPorB + CpG-1826, respectively. Lane 8) *C. trachomatis* MoPn EB. Lane 9) MEM-0. Lane 10) Pre-immunization sera.

Figure 2. Changes in body weight following the i.n. challenge with *C. trachomatis*

A. Daily percentage change in mean body weight following the i.n. *C. trachomatis* challenge. *, $p < 0.001$ as determined by the Repeated Measures ANOVA.

B. Percentage change in mean body weight of each group at day 10 after the i.n. *C. trachomatis* challenge. The mean is indicated as a short line. Each symbol represents a single animal.

Figure 3. Disease burden and *Chlamydia* yields from the lungs at day 10 following the i.n. *C. trachomatis* challenge

A. Lungs weight (g) at day 10 after the i.n. *C. trachomatis* challenge. The mean is shown as a short line. Each symbol represents a single animal.

B. Number of *Chlamydia* IFU recovered from the lungs at day 10 after the i.n. challenge. The median is shown as a short line. Each symbol represents a single animal.

Table 1

Pilot experiment: antibody response and disease burden.

Adjuvants	Dose $\mu\text{g}/\text{immunization}$	TLR/NOD	IgG2a/IgG1	% change in body weight		Lung weight (g)		#IFU recovered from lungs	
				Mean $\pm$ ISE	Mean $\pm$ ISE	Mean $\pm$ ISE	Median, (Min - Max)	Median, (Min - Max)	Median, (Min - Max) $\times 10^6$
Pam ₂ CSK ₄	5	TLR2/6	0.004	-4.7 $\pm$ 1.9	0.26 $\pm$ 0.02	0.26 $\pm$ 0.02	15 (0.7–314)	15 (0.7–314)	
Poly (I:C)	10	TLR3	1	-9.8 $\pm$ 1.5	0.26 $\pm$ 0.02	0.26 $\pm$ 0.02	13 (3 – 2,053)	13 (3 – 2,053)	
Imidazoquinoline R848	10	TLR7/8	0.016	-15.8 $\pm$ 3.4	0.24 $\pm$ 0.01	0.24 $\pm$ 0.01	281 (24 – 12,080)	281 (24 – 12,080)	
M-Tri-DAP	10	NOD1/2	0.031	-14.1 $\pm$ 3.1	0.28 $\pm$ 0.03	0.28 $\pm$ 0.03	333 (2 – 2,941)	333 (2 – 2,941)	
Muramyl dipeptide	10	NOD2	0.008	-12.7 $\pm$ 1.8	0.22 $\pm$ 0.03	0.22 $\pm$ 0.03	195 (144 – 303)	195 (144 – 303)	
Imiquimod R837	10	TLR7	0.016	-12.9 $\pm$ 2.5	0.25 $\pm$ 0.02	0.25 $\pm$ 0.02	23 (0.004 – 2,516)	23 (0.004 – 2,516)	
Flagellin (B. subtilis)	5	TLR5	0.016	-13.2 $\pm$ 2.2	0.30 $\pm$ 0.04	0.30 $\pm$ 0.04	170 (14 – 2,941)	170 (14 – 2,941)	
Monophosphoryl lipid A	5	TLR4	1	-14.3 $\pm$ 2.3	0.30 $\pm$ 0.02	0.30 $\pm$ 0.02	1,831 (1,527 – 2,849)	1,831 (1,527 – 2,849)	
CpG-1826	10	TLR9	8	-11.5 $\pm$ 2.0	0.27 $\pm$ 0.02	0.27 $\pm$ 0.02	809 (17 – 4,421)	809 (17 – 4,421)	
None*	---	---	---	-18.5 $\pm$ 1.8	0.30 $\pm$ 0.02	0.30 $\pm$ 0.02	851 (3,731 – 9,966)	851 (3,731 – 9,966)	

* , None, the animals were inoculated i.n. once with MEM-0.

Table 2

Experimental groups.

Vaccine		Amount/mouse/dose		Routes of immunizations		No. of mice
Antigen	Adjuvant	Antigen	Adjuvant			
Ct-rMOMP	Pam ₂ -CSK ₄	10µg	5µg	i.m.	3	26
Ct-rMOMP	Poly (I:C)	10µg	10µg	i.m.	3	21
Ct-rMOMP	CpG-1826	10µg	10µg	i.m.	3	27
Ng-rPorB	Pam ₂ -CSK ₄	10µg	5µg	i.m.	3	10
Ng-rPorB	Poly (I:C)	10µg	10µg	i.m.	3	10
Ng-rPorB	CpG-1826	10µg	10µg	i.m.	3	27
Live EB	---	10 ⁴ IFU	---	i.n.	1	26
MEM-0	---	---	---	i.n	1	27

Table 3
Serum antibody GMT (range) the day before the i.n. challenge with *Chlamydia* MoPn.

Vaccine	IgG	IgG1	IgG2a	IgG2a/IgG1	IgA
Ct-rMOMP + Pam ₂ CSK ₄	64,508 (51,200–102,400)	16,127 (12,800–25,600)	400 (100–6,400)	0.02	8,063 (6,400–12,800) ^a
Ct-rMOMP + Poly (I:C)	32,254 (25,600–51,200)	1,600 (800–6,400)	2,540 (1,600–3,200)	1.6	1,600 (1,600–1,600)
Ct-rMOMP + CpG-1826	40,637 (25,600–51,200)	400 (400–400)	2,540 (1,600–3,200)	6.4	800 (400–1,600)
Ng-rPorB + Pam ₂ CSK ₄	<100	<100	<100	---	<100
Ng-rPorB + Poly (I:C)	<100	<100	<100	---	<100
Ng-rPorB + CpG-1826	<100	<100	<100	---	<100
Live EB	51,200 (51,200–51,200)	800 (800–800)	20,319 (12,800–25,600)	25.4	4,032 (3,200–6,400)
MEM-0	<100	<100	<100	---	<100

^a, $p < 0.05$ by the Student's t -test compared with the groups immunized with Ct-rMOMP + Poly (I:C) or Ct-rMOMP + CpG-1826.

In vitro T cell proliferative response and cytokine production from stimulated T cells of immunized mice the day before the i.n. challenge with *Chlamydia* MoPn.

Table 4

Vaccine	Routes	cpm ± 1SE		SI [*]	IFN-γ (mean ± 1SE, pg/ml)		IL-10 (mean ± 1SE, pg/ml)		IL-4 (mean ± 1SE, pg/ml)	
		EB	Medium		EB	Medium	EB	Medium	EB	ConA
Ct-rMOMP+Pam ₂ CSK ₄	i.m.	6,653 ± 82 ^a	295 ± 17	23	9,149 ± 1,896 ^c	<31	105 ± 7	<31	18.6 ± 1.0	118.9 ± 9.1
Ct-rMOMP+Poly (I:C)	i.m.	5,101 ± 71 ^b	207 ± 14	25	1,532 ± 240 ^d	<31	151 ± 18	<31	<7.8	70.7 ± 11.8
Ct-rMOMP+CpG-1826	i.m.	3,544 ± 60	262 ± 16	14	1,161 ± 60 ^e	<31	142 ± 22	<31	<7.8	129.5 ± 21.7
Ng-rPorB+Pam ₂ CSK ₄	i.m.	1,327 ± 36	160 ± 13	8	301 ± 15	<31	92 ± 12	<31	<7.8	150.9 ± 33.1
Ng-rPorN+Poly (I:C)	i.m.	641 ± 25	345 ± 19	2	242 ± 16	<31	78 ± 10	<31	<7.8	154.7 ± 25.2
Ng-rPorB+CpG-1826	i.m.	1,169 ± 34	119 ± 11	10	481 ± 8	<31	124 ± 12	<31	<7.8	107.3 ± 8.8
10,000 live EB	i.n.	7,503 ± 87	166 ± 13	45	23,639 ± 2,506	<31	220 ± 18	<31	22.6 ± 1.9	97.6 ± 14.3
MEM-0	i.n.	646 ± 25	193 ± 14	3	263 ± 18	<31	114 ± 8	<31	<7.8	118.3 ± 8.1

^a, *p* < 0.05 by the Student's *t*-test compared with the groups immunized with Ct-rMOMP + CpG-1826, or Ng-rPorB + Pam₂CSK₄, or MEM-0.

^b, *p* < 0.05 by the Student's *t*-test compared with the groups immunized with Ct-rMOMP + CpG-1826, or Ng-rPorB + Poly (I:C), or MEM-0.

^c, *p* < 0.05 by the Student's *t*-test compared with the groups immunized with Ct-rMOMP + Poly (I:C), or Ct-rMOMP + CpG-1826, or Ng-rPorB + Pam₂CSK₄, or MEM-0.

^d, *p* < 0.05 by the Student's *t*-test compared with the groups immunized with Ng-rPorB + Poly (I:C), or MEM-0.

^e, *p* < 0.05 by the Student's *t*-test compared with the groups immunized with Ng-rPorB + CpG-1826, or MEM-0.

^{*}, SI = Stimulation index.

Table 5

Disease burden and yields of *Chlamydia* MoPn from the lungs at day 10 p.c.

Vaccine	Route	% change in body weight (Mean $\pm$ 1SE)	Lungs weight (g) (Mean $\pm$ 1SE)	No. IFU recovered from lungs Median (Min-Max), $\times 10^6$
Ct-rMOMP + Pam ₂ CSK ₄	i.m.	-4.8 $\pm$ 0.8 ^a	0.27 $\pm$ 0.01 ^d	6 (0.04 – 314) ^e
Ct-rMOMP + Poly (I:C)	i.m.	-11.3 $\pm$ 1.2 ^b	0.27 $\pm$ 0.01 ^b	26 (3 – 2,053) ^f
Ct-rMOMP + CpG-1826	i.m.	-11.0 $\pm$ 1.2 ^c	0.28 $\pm$ 0.01 ^c	28 (0.009 – 4,421) ^g
Ng-rPorB + Pam ₂ CSK ₄	i.m.	-13.7 $\pm$ 1.7	0.26 $\pm$ 0.02	117 (0.9 – 1,628)
Ng-rPorB + Poly (I:C)	i.m.	-20.6 $\pm$ 1.6	0.35 $\pm$ 0.01	241 (22 – 4,995)
Ng-rPorB + CpG-1826	i.m.	-17.7 $\pm$ 0.9	0.32 $\pm$ 0.01	185 (5 – 8,695)
10,000 live EB	i.n.	0.7 $\pm$ 0.6	0.20 $\pm$ 0.01	BLD [*]
MEM-0	i.n.	-17.5 $\pm$ 1.1	0.32 $\pm$ 0.01	264 (6 – 9,966)

^a, $p < 0.05$ by the Student's t -test compared with the group immunized with Ct-rMOMP + Poly (I:C), Ct-rMOMP + CpG-1826, Ng-rPorB + Pam₂CSK₄, or MEM-0.

^b, $p < 0.05$ by the Student's t -test compared with the group immunized with Ng-rPorB + Poly (I:C) or MEM-0.

^c, $p < 0.05$ by the Student's t -test compared with the group immunized with Ng-rPorB + CpG-1826 or MEM-0.

^d, $p < 0.05$ by the Student's t -test compared with the group immunized with MEM-0.

^e, $p < 0.01$ by the Mann Whitney's U -test compared with the groups immunized with Ct-rMOMP + Poly (I:C), Ct-rMOMP + CpG-1826, Ng-rPorB + Pam₂CSK₄, or MEM-0.

^f, $p < 0.01$ by the Mann Whitney's U -test compared with the groups immunized with Ng-rPorB + Poly (I:C) or MEM-0.

^g, $p < 0.01$ by the Mann Whitney's U -test compared with the groups immunized with Ng-rPorB + CpG-1826 or MEM-0.

^{*} BLD: below limit of detection: 50 IFU/lungs/mouse