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A vaccine formulated with a combination of TLR-2 and TLR-9 adjuvants and the recombinant major outer membrane protein elicits a robust immune response and significant protection against a *C. muridarum* challenge

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Abstract

Chlamydia trachomatis is the most common sexually transmitted bacterial pathogen in the World and there is a need for a vaccine. To enhance the immunogenicity of a vaccine formulated with the *Chlamydia muridarum* (Cm) mouse pneumonitis recombinant major outer membrane protein (MOMP), we used combinations of Pam_2CSK_4+CpG-1826 and Montanide ISA 720 VG +CpG-1826 as adjuvants. *Neisseria gonorrhoeae* recombinant porin B (Ng-PorB) was used as the antigen control with the same adjuvants. Female BALB/c mice were primed twice in the nares (i.n.) or in the colon (cl.) and were boosted twice by the intramuscular plus subcutaneous (i.m. +s.c.) routes. Based on the IgG2a/IgG1 ratio in sera, mice immunized with MOMP +Pam_2CSK_4+CpG-1826 showed a strong Th2 response while animals vaccinated with MOMP +Montanide ISA 720 VG+CpG-1826 had a Th1 response. Both groups of mice also developed robust Cm-specific T cell proliferation and high levels of IFN-γ. Four weeks after the last immunization, the mice were challenged i.n. with 10^4 inclusion-forming units (IFU) of Cm. Using changes in body weight and number of IFU recovered from the lungs at 10 days post-challenge mice immunized i.n.+i.m./s.c. with MOMP+Pam_2CSK_4+CpG-1826 were better protected than other groups. In conclusion, MOMP adjuvanted with Pam_2CSK_4+CpG-1826, elicits strong humoral and cellular immune responses and induces significant protection against *Chlamydia*.

Keywords

Chlamydia trachomatis; *Chlamydia muridarum*; vaccine; routes of immunization; Toll-like receptors; CpG-1826; Montanide ISA 720 VG; Pam_2CSK_4

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1. Introduction

C. trachomatis is worldwide the leading cause of bacterial sexually transmitted diseases and can also produce respiratory, gastrointestinal and ocular infections. Genital infections affect particularly young sexually active individuals [1–4]. Newborns become infected in the birth canal and contract ocular and respiratory infections [2, 4, 5]. Adult immunocompromised individuals can also suffer from respiratory infections [6]. Antibiotic therapy is available but, due to the high percentage of asymptomatic patients, or inappropriate treatment, long-term sequelae can develop including abdominal pain, infertility, ectopic pregnancy and blindness [3, 4, 7, 8]. Countries that have established screening programs for genital infections, followed by antibiotic therapy, have observed an increase in the prevalence of the infection [9, 10]. This increase is thought to be due to a block in the development of natural immunity as a result of the antibiotic therapy [9]. Thus, a vaccine will be the best approach to control chlamydial infections [11–13].

Current vaccines, formulated with live or inactivated whole pathogens, possess intrinsic adjuvant activity as they contain “pathogen-associated molecular patterns” (PAMPs), such as LPS and nucleic acids that activate in the host immune cells “pattern recognition receptors” (PRRs), such as toll-like receptors (TLR) [14, 15]. Therefore, the vaccinated individual, upon exposure to the pathogen, is ready to mount a robust immune response. The use of highly purified antigens, including synthetic peptides or recombinant proteins that, for the most part, lack inherent adjuvanticity, has resulted in the need to develop adjuvants to enhance the immune responses [16–18].

Several TLR agonists were recently screened individually for their ability to serve as adjuvants in a vaccine formulated with the *Chlamydia. muridarum* (Cm) recombinant major outer membrane protein (MOMP) [19]. It was found that Pam_2CSK_4, a TLR2 ligand, and CpG-1826, a ligand for TLR9, were effective adjuvants for enhancing protection against a chlamydial respiratory challenge [19–21]. Protection against a secondary chlamydial infection is dependent on CD4 Th1 cells, B cells and antibodies [22]. CpG-1826 elicits a strong Th1 response while Pam_2CSK_4 enhances antibody responses [23, 24]. Therefore, here we tested a vaccine with recombinant MOMP and CpG-1826+Pam_2CSK_4, or CpG-1826+Montanide ISA 720 VG as adjuvants, to determine which combination elicits the most robust protection against a *C. muridarum* challenge [25].

2. Materials and methods

2.1. Chlamydia muridarum stocks

The *C. muridarum* [Cm; previously called *C. trachomatis* mouse pneumonitis (MoPn) strain Nigg II], was grown in HeLa-229 cells and elementary bodies (EB) were purified as described [26].

2.2. Cloning and purification of the recombinant C. muridarum MOMP and the N. gonorrhoeae porin B (Ng-PorB)

Genomic DNA from *C. muridarum* and *N. gonorrhoeae* strain FA 1090 (ATCC) were extracted using the Wizard genomic DNA Purification Kit (Promega, Madison, WI) [27]. The Cm MOMP (GenBank, accession no. AE002272, X63409) and Ng-PorB genes (36 kDa, GenBank ID: AAW90430) were amplified as previously described and *E. coli* BL21 (DE3) competent cells were used for protein expression [27]. The recombinant proteins were purified using a Sephacryl-S-300 column [25, 27, 28].

The purity of MOMP and Ng-PorB was determined by 10% tricine-SDS-PAGE [29]. Using the limulus amoebocyte assay (BioWhittaker, Inc., Walkersville, MD), both proteins were found to have less than 0.05 EU of LPS/mg of protein.

2.3. 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 UCI, Animal Care and Use Committee approved all animal protocols.

Agonists of TLR2 (Pam_2CSK_4, InvivoGen, San Diego, CA; 5 μg/mouse/immunization) and TLR9 (CpG-1826, TriLink, San Diego, CA; 10 μg/mouse/immunization) were directly mixed with the antigens (10 μg/mouse/immunization) as published [19, 26]. The TLR-independent adjuvant Montanide ISA 720 VG (Seppic Inc, Fairfield, NJ; at a concentration of 70% of the total volume of the vaccine formulation) was also used. CpG-1826 (C) and Pam_2CSK_4 (P) were delivered by the mucosal and systemic routes while Montanide ISA 720 VG was only used systemically (M^s) since it is not a mucosal adjuvant [30].

Following anesthesia mice (n=14 to 20) were vaccinated according to Schedule 1: twice by a mucosal route [intranasal (i.n.)] followed twice systemic routes [intramuscular plus subcutaneous (i.m./s.c.)] or Schedule 2: twice colonic (cl.) followed twice i.m./s.c. All immunizations were given at 2-week intervals (Table 1). As a negative antigen control, mice were vaccinated with the Ng-PorB using the same adjuvants, delivery routes and immunization schedules. The antigens were equally divided among three sites: bilateral quadrices femoris muscles (i.m.) and base of the tail (s.c.). The positive control mice were immunized i.n. once with 10^4 inclusion forming units (IFU) of Cm in 20 μl of Eagle's MEM without serum [(MEM-0); Invitrogen, Carlsbad, CA]. A negative control group was inoculated once i.n. with 20 μl MEM-0 [31]. These last two control groups were immunized once at the same time when the other animals received their first immunization. All animals were of the same age at the time of challenge.

2.4. Characterization of the humoral and cell mediated immune responses

To measure Cm-specific antibodies, blood was collected from each mouse the day before the challenge. ELISA and the Western blot were performed as previously described with Cm EB as the antigen [25].

To assess the T cell memory responses an in vitro lymphoproliferative assay was performed before the i.n. challenge as previously described [31, 32]. 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) [33].

2.5. Intranasal challenge

After the last immunization the animals were rested for 4 weeks and then challenged i.n. with 10^4 IFU of Cm under Xylazine/Ketamine anesthesia [34]. At day 10 post-challenge (p.c.) the mice were euthanized, their lungs homogenized in 5 ml of SPG, and serial 10-fold dilutions were inoculated onto Hela-229 cells. The chlamydial inclusions were stained with a cocktail of monoclonal antibodies and were counted [32]. The limit of detection per pair of lungs was 50 IFU. To determine the local humoral and cellular immune responses the titers of Cm-specific IgA and levels of IFN-γ in lungs at D10 p.c. were determined by an ELISA, as described above.

2.6. Statistical analyses

Statistical analyses were performed with the SigmaStat and SAS software. 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. A pairwise Pearson's correlation analysis was performed to investigate the association between the observations of interest [31]. Differences were considered significant for values of $p \leq 0.05$.

3. Results

3.1. Humoral immune responses following vaccination

Mice immunized with MOMP/P/C developed a Th2-biased response, independent of the routes of vaccination used, as shown by higher geometric mean titers (GMT) of IgG1 in comparison to IgG2a (IgG1=25,600 and IgG2a=2540, IgG2a/IgG1=0.10 for MOMP/P/C-1 immunization; IgG1=81,275 and IgG2a=16,127, IgG2a/IgG1=0.20 for MOMP/P/C-2 immunization; Table 1). In contrast, mice immunized with MOMP/M^s/C mounted a Th1 response (IgG1=16,127 and IgG2a=51,200, IgG2a/IgG1=3.17 for MOMP/M^s/C-1 immunization; IgG1=32,254 and IgG2a=102,400, IgG2a/IgG1=3.17 for MOMP/M^s/C-2 immunization). Live EB elicited a strong Th1-based response (IgG1=1600 and IgG2a=12,800, IgG2a/IgG1=8). Mice vaccinated with MOMP/P/C-1 and MOMP/M^s/C-1 showed high serum IgA titers (IgA=1008 and IgA=4032) compared to those immunized with MOMP/P/C-2 and MOMP/M^s/C-2 (IgA=504 and IgA=800). The differences for the IgA titers were not statistically significant among the groups immunized with MOMP or with live EB (IgA=2016).

The Western blot shows that mice vaccinated with the MOMP developed antibodies only against this protein (MW 40 kDa; Supplemental Fig. 1). Animals immunized i.n. with EB 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 and the 28-kDa protein.

3.2. Cell mediated immune responses following vaccination

Overall, the response of splenic T-cells, stimulated with UV-inactivated EB, from mice immunized with MOMP, was significantly stronger than their respective negative controls immunized with Ng-PorB or MEM-0 (Fig. 1A, $p<0.05$). The proliferative response in mice immunized with MOMP/P/C-1 [2870±99 cpm, stimulation index (SI=cpm from EB stimulation/cpm from medium stimulation) =15] was similar to the response of mice immunized with MOMP/P/C-2 (2612±217 cpm, SI=11). A similar response was also found in mice immunized with MOMP/M^s/C-1 (3042±249 cpm, SI=13) in comparison to animals immunized with MOMP/M^s/C-2 (2423±220 cpm, SI=11) ($p > 0.05$). The most robust lymphoproliferative response was observed in control mice immunized i.n. with EB (6119±530 cpm, SI=29).

The level of IFN-γ (Fig. 1B) produced by EB-stimulated splenic T-cells of mice immunized with MOMP/M^s/C-2 (433±55 pg/ml) was significantly higher than that from mice immunized with MOMP/M^s/C-1 (176±10 pg/ml, $p < 0.05$). No significant difference was found between mice immunized with MOMP/P/C-2 (377±138 pg/ml) and those immunized with MOMP/P/C-1 (135±17 pg/ml; $p>0.05$). The highest level of IFN-γ was detected in supernatants of activated T cells from mice vaccinated i.n. with EB (18,142±3808 pg/ml). This level was significantly higher in comparison to any group immunized with MOMP ($p<0.05$).

3.3. Changes in body weight following the i.n. challenge

All mice, except those immunized i.n. with EB, lost weight for the first 4 days p.c. (Fig. 2A). Afterward, mice immunized with MOMP regained or maintained the weight up to the end of the observation at D10 p.c. Mice immunized with MOMP/P/C-1 lost less body weight during the 10 days than mice immunized with MOMP/P/C-2, MOMP/M^s/C-1 or MOMP/M^s/C-2 (p<0.05 by repeated measured ANOVA). In contrast, mice immunized with Ng-PorB or MEM-0 consistently lost body weight during the 10 days of observation. Animals immunized with Ng-PorB/M^s/C-1 showed significant less body weight loss over the 10 days of observation in comparison to other negative control groups (p<0.05 by repeated measured ANOVA) probably reflecting a non-specific protection.

As shown in Fig. 2B and Table 2, at D10 p.c., control mice inoculated i.n. with MEM-0 and those immunized with Ng-PorB had lost significantly more body weight in comparison to animals immunized with MOMP (p<0.05). Mice immunized with MOMP/P/C-1 had only lost 3.12±0.85% of their initial mean body weight. However, no significant differences were found among this group and the other three groups of mice immunized with MOMP/P/C-2 (6.03±1.50%), MOMP/M^s/C-1 (5.35±0.78%) or MOMP/M^s/C-2 (5.11±1.01%) (p>0.05).

3.4. Burden of C. muridarum infection in the lungs

The number of Cm IFU from the lungs of mice immunized with MOMP was significantly lower than from animals inoculated i.n. with MEM-0 or with Ng-PorB (p<0.05); (Fig. 2C; Table 2). The median number of IFU recovered from lungs of mice vaccinated with MOMP/P/C-1 was 0.74 [range: below limit of detection (BLD)–15] × 10^6 IFU. The number of IFU in this group was significantly less than in mice immunized with MOMP/P/C-2 [0.66 (range: 0.37–177.6) × 10^6 IFU; p<0.05], or from mice immunized with MOMP/M^s/C-1 [1.6 (range: BLD–21.5) × 10^6 IFU; p=0.05]. In contrast, the median number of IFU recovered from lungs of mice immunized with MOMP/M^s/C-2 [0.14 (range:<0.02–146) × 10^6 IFU] was not significantly different than in mice immunized with MOMP/M^s/C-1 (p>0.05), or mice immunized by the same routes with MOMP/P/C-2 [0.66 (range: 0.37–177.6) × 10^6 IFU; p>0.05]. The difference in the number of IFU recovered from mice immunized with MOMP/P/C-1 and animals immunized with MOMP/M^s/C-2 was approaching statistical significance (p=0.069).

3.5. Immune responses in lungs at 10 days p.c

The levels of Cm-specific IgA in the lungs of mice inoculated i.n. with MEM-0 or Ng-PorB were significantly lower than in any other group immunized with MOMP (p<0.05; Fig. 3A; Table 2). The mean OD_{405nm} of Cm-specific IgA in mice immunized with MOMP/P/C-1 was 1.699±0.177. This was significantly higher than that of mice immunized with MOMP/P/C-2 (0.809±0.032), MOMP/M^s/C-1 (0.496±0.017) or MOMP/M^s/C-2 (0.674±0.039) (p<0.05). Furthermore, the level of Cm-specific IgA in mice immunized with MOMP/P/C-2 was statistically higher than in mice immunized with MOMP/M^s/C-2 (p<0.05). As expected, mice inoculated i.n. with EB showed the highest level of Cm-specific IgA (2.315±0.083) in the lungs in comparison to any other group (p<0.05).

The levels of IFN-γ detected in lungs at 10 days p.c. are shown in Fig. 3B and Table 2. Overall, there was an inverse correlation between the levels of IFN-γ and those of Cm-specific IgA. Mice immunized with MOMP/P/C-1, MOMP/P/C-2, MOMP/M^s/C-1 or MOMP/M^s/C-2 showed significantly lower level of IFN-γ (387±169 pg/ml; 584±151 pg/ml; 1875±418 pg/ml; 201±40 pg/ml) in comparison to their respective controls immunized with Ng-PorB and the same adjuvants (12,620±2160 pg/ml; 5736±1872 pg/ml; 11,557±2285 pg/ml; 2686±429 pg/ml; p<0.05). As expected, the lowest level of IFN-γ was detected in mice inoculated i.n. with live EB (94.4±11.9 pg/ml; p<0.05). The relatively low level of IFN-γ in

mice inoculated with MEM-0 (1929±315 pg/ml) in comparison to those immunized with Ng-PorB, is likely a reflection of the severity of the Cm infection.

3.6. Correlation analyses between the observations

Pairwise Pearson's correlation analysis was performed and showed that the five determinations: in vitro splenic T-cell proliferative responses (cpm) before challenge, percentage change in mean body weight p.c., number of Cm IFU recovered from the lungs at D10 p.c., and the levels of Cm-specific IgA (OD_{405nm}) and IFN-γ (pg/ml) in the lungs at 10 days p.c., were significantly correlated to each other ($p<0.05$; Table 3).

4. Discussion

In this study we compared the protective efficacy of vaccines formulated with the MOMP using P/C, or M^s/C, as adjuvants delivered by a combination of mucosal and systemic routes. Robust humoral and cellular immune responses were elicited in BALB/c by all vaccine formulations. As determine by the local immune response in the lungs, body weight changes and number of IFU recovered from lungs, mice vaccinated with MOMP/P/C-1 had a more robust local immune response and were better protected against a respiratory challenge with Cm than the other groups.

Optimally adjuvants used for vaccination should utilize the same signaling pathways that the pathogen will activate at the time of a natural infection. During a *Chlamydia* infection TLR and Nod 1 mediated activation occurs [35–38]. Several chlamydial PAMPs, including LPS, MOMP, macrophage infectivity potentiator (Mip), heat shock protein-60 (hsp60) and peptidoglycan are involved in this process resulting in signaling mainly through TLR2 and TLR4 while the role of TLR-9 in chlamydial infections is under investigation [39, 40]. Thus, to enhance the immune response of a vaccine formulated with MOMP, we tested a combination of Pam_2CSK_4, a TLR-2 and CpG-1826, a TLR-9, adjuvants [18, 19, 41, 42].

TLR expression varies depending on the type of tissues and therefore, TLR agonists produce different responses depending upon the route of administration [43]. Protection against a chlamydial genital challenge is enhanced by combining mucosal and systemic routes for immunization [44]. For these reasons here, BALB/c mice were vaccinated using combinations of mucosal (Schedule 1: i.n. or Schedule 2: cl.) and systemic routes (i.m/s.c.) to determine if they will elicit robust immune responses and protection against a respiratory challenge. Up to now, of the mucosal routes, i.n. immunization has been shown to be the best approach for inducing protection against respiratory and genital chlamydial challenges [32, 45]. The use of the i.n. route for immunization however, can result in significant negative secondary effects [46]. For these reasons, we decided to also explore the cl. route [47, 48]. Colonic immunization may be an appropriate vaccination target since it has been shown to induce higher vaginal IgA levels than oral and even i.m. immunization, as well as higher serum IgA levels than the oral route [48]. This may be highly relevant for sexually transmitted infections like *C. trachomatis* since the colon is a source of IgA plasma effector for the vaginal tract [48].

Both adjuvants formulations elicited high Cm-specific antibody titers. As determined by the IgG2a/IgG1 ratio, animals vaccinated with MOMP/P/C had a Th2-biased immune response while the groups immunized using MOMP/M^s/C had strong Th1 responses. This confirms previous findings showing that, based on the IgG2a/IgG1 ratio in sera, Pam_2CSK elicits a Th2 biased immune response. Interestingly, Pam_2CSK stimulated the production of IFN-γ in T lymphocytes indicative of a mixed Th1/Th2 immune response [49]. Similar results were obtained following i.n. vaccination of BALB/c mice with the recombinant HIV-1 p17 protein adjuvanted with synthetic MALP-2 [50]. This formulation, as determined by IgG2a/

IgG1 ratio, elicited a Th2-biased immune response but a Th1-biased cellular response based on the frequency of IFN-γ-secreting spleen cells. MALP-2, like Pam_2CSK, is a diacylated lipopeptide that also signal through TLR-2 and therefore, both compounds likely have similar adjuvant properties.

Following the i.n. challenge, the changes in body weight and number of MoPn IFU recovered from the lungs demonstrated that mice immunized with MOMP/P/C were better protected than the other groups. Also, this protection was more robust than that previously reported when using formulations with single adjuvants [19]. Furthermore, these parameters of protection correlated with the levels of Cm-specific IgA and IFN-γ in the lungs. Significantly higher levels of Cm-specific IgA were detected in the lungs of mice immunized with MOMP/P/C in comparison to animals immunized with MOMP/M^s/C. This was more evident in mice vaccinated using i.n. priming rather than cl. indicating that i.n. immunization is very effective at eliciting strong local humoral immune responses in the lungs. These results are similar to those observed with MALP-2 that also induced high levels of p17-specific IgA in the lungs and vaginal washes in BALB/c mice vaccinated with this recombinant HIV-1 protein [50]. Furthermore, lower levels of IFN-γ, indicative of resolution of the infection at 10 days p.c., were detected in the lungs of the mice immunized with MOMP/P/C-1 in comparison to the mice vaccinated with MOMP/M^s/C-1.

In conclusion, MOMP formulated with Pam_2CSK_4 plus CpG-1826 and delivered by a combination of i.n. and i.m.+s.c. routes (MOMP/P/C-1), induced in mice strong humoral and cell mediated immune responses and the animals showed a robust protection against a respiratory challenge with *C. muridarum*. Now it will be interesting to test this vaccine formulation against a genital challenge. Comparing the i.n. versus the cl. routes of vaccination will be important since the regional lymph nodes of the genital tract are in close proximity and may be shared, with those of the gastrointestinal system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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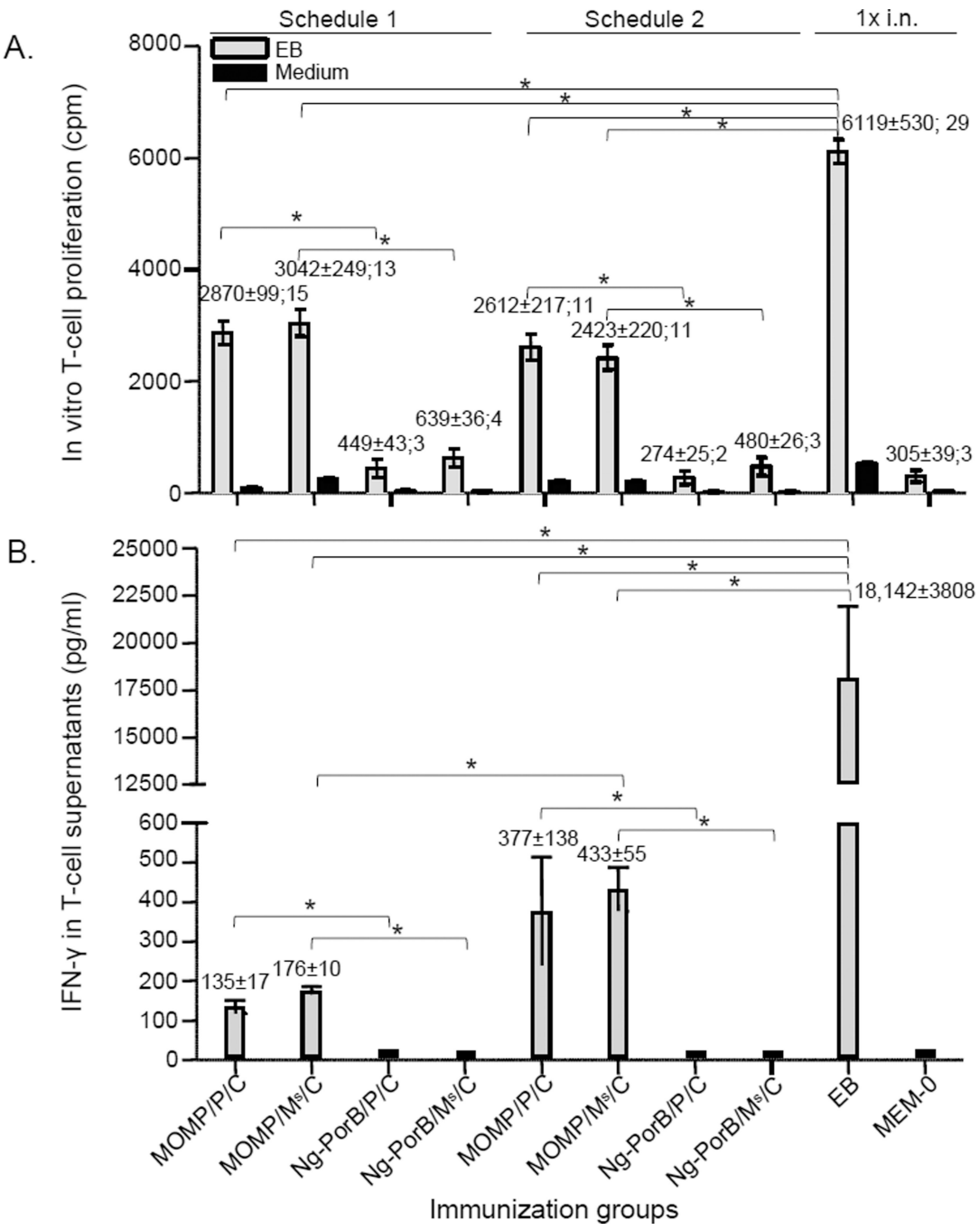

Fig. 1. In vitro splenic T-cell responses before the i.n. challenge

A. Splenic T-cell proliferation (mean cpm ± 1SE; SI). T-cells were stimulated with UV-treated Cm EB, or medium as a control, the day before the i.n. Cm challenge. *, $p < 0.05$ as determined by the two-tailed unpaired Student's *t*-test.

B. Levels of IFN-γ (mean pg/ml ± 1SE) detected in supernatants of proliferating splenic T-cells stimulated with UV-treated Cm EB. *, $p < 0.05$ as determined by the two-tailed unpaired Student's *t*-test.

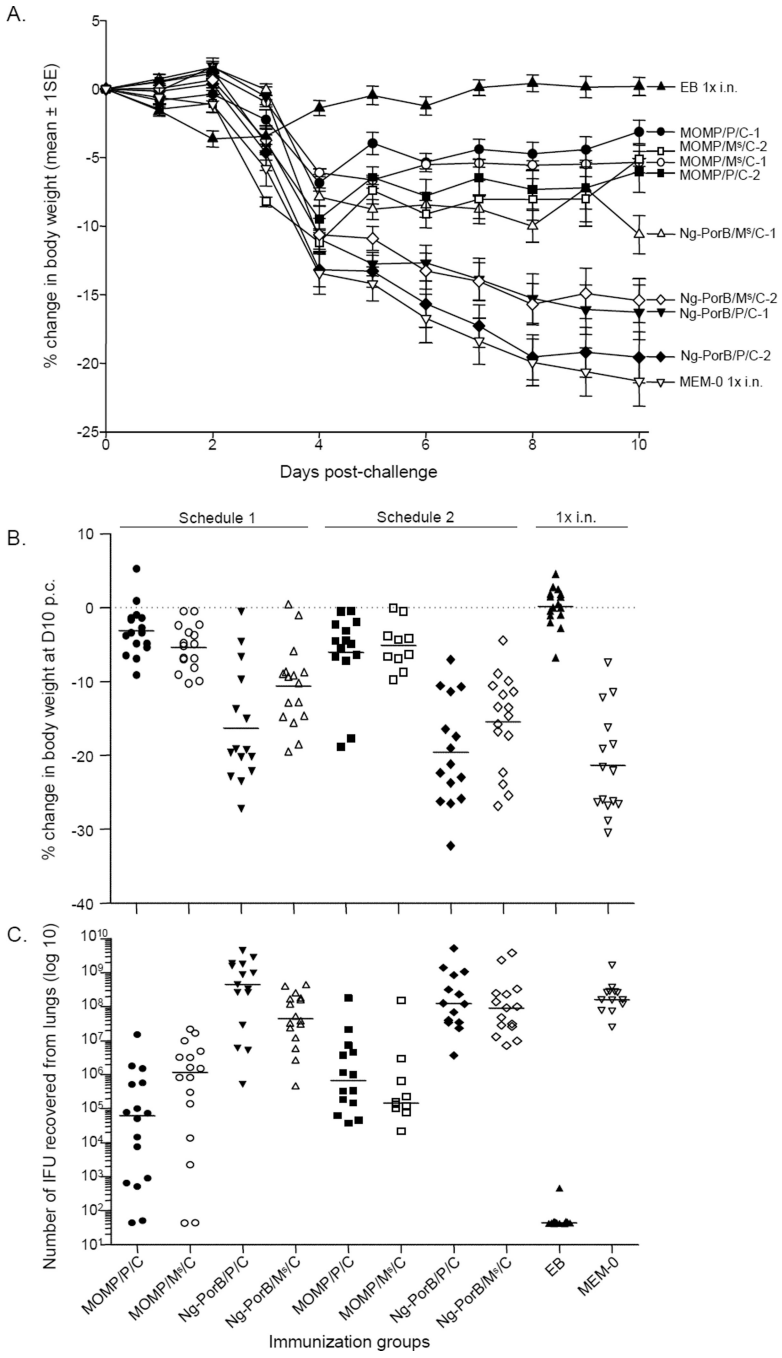

Fig. 2. Changes in body weight and Cm yields from the lungs at day 10 following the i.n. challenge

A. Percentage change in body weight of each group following the i.n. Cm challenge.

B. Percentage change in body weight at day 10 after the i.n. Cm challenge. The mean is indicated as a horizontal line. Each symbol represents a single animal.

C. Number of Cm IFU recovered from the lungs at day 10 after the i.n. challenge. The median is shown as a horizontal line. Each symbol represents a single animal.

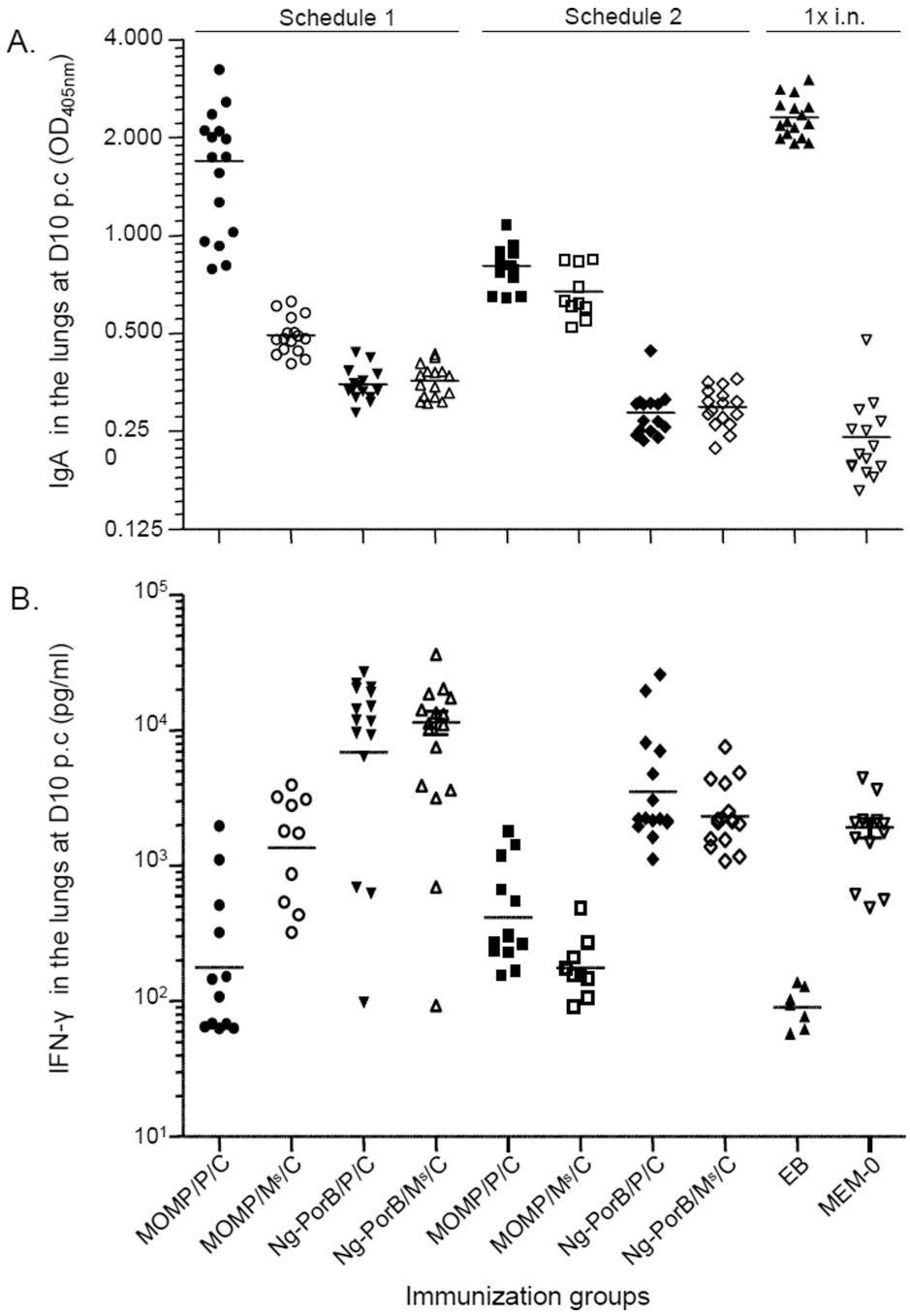

Fig. 3. Levels of Cm-specific IgA and IFN-γ detected in the lungs at day 10 following the i.n. challenge

A. Levels of Cm-specific IgA (405nm OD) detected in the lungs at day 10 after the i.n. Cm challenge. The mean is shown as a horizontal line. Each symbol represents a single animal.
B. Levels of IFN-γ (pg/ml) detected in the lungs at day 10 after the i.n. Cm challenge. The mean is shown as a horizontal line. Each symbol represents a single animal.

Table 1

Serum antibody geometric mean titers (GMT; range) the day before the i.n. challenge with *C. muridarum*.

Vaccine	Routes and number of immunizations	No. of mice	IgG1	IgG2a	IgG2a/ IgG1	IgA
MOMP/P/C-1	2× i.n. + 2× i.m./s.c.	20	25,600 (12,800 – 51,200)	2540 (1600 – 3200)	0.10	1008 (800 – 1600)
MOMP/M^{s}/C-1	2× i.n. + 2× i.m./s.c.	20	16,127 (6400 – 25,600)	51,200 (25,600 – 102,400)	3.17	4032 (3200 – 6400)
Ng-PorB/P/C-1	2× i.n. + 2× i.m./s.c.	19	<100	<100	---	<100
Ng-PorB/M^{s}/C-1	2× i.n. + 2× i.m./s.c.	20	<100	<100	---	<100
MOMP/P/C-2	2× cl. + 2× i.m./s.c.	17	81,275 (51,200 – 102,400)	16,127 (12,800 – 25,600)	0.20	504 (400 – 800)
MOMP/M^{s}/C-2	2× cl. + 2× i.m./s.c.	14	32,254 (25,600 – 51,200)	102,400 (51,200 – 204,800)	3.17	800 (200 – 1600)
Ng-PorB/P/C-2	2× cl. + 2× i.m./s.c.	19	<100	<100	---	<100
Ng-PorB/M^{s}/C-2	2× cl. + 2× i.m./s.c.	20	<100	<100	---	<100
EB	1 × i.n.	20	1600 (800 – 3200)	12,800 (6400 – 25,600)	8	2016 (1600 – 3200)
MEM-0	1 × i.n.	19	<100	<100	---	<100

P: Pam$_2$CKS$_4$

C: CpG-1826

M^{S}: Montanide was only used by systemic routes (im./sc.)

1: Schedule 1= 2× in. + 2× im./sc.

2: Schedule 2= 2× cl. + 2× im./sc.

Table 2

Disease burden, yields of *C. muridarum* IFU recovered from the lungs and Cm-specific IgA and IFN-γ in the lungs at day 10 p.c.

Vaccine	D10 BWC^ (%) Mean ± 1SE	IFN-γ in lungs (pg/ml) Mean ± 1SE	IgA in lungs (OD_{405nm}) Mean ± 1SE	#IFU in lungs Median (Min-Max) ×10^6
MOMP/P/C-1	−3.12 ± 0.85[a]	387 ± 169[a, e]	1.699 ± 0.177[a, e, f]	0.74 (BLD* – 15)[h, i, j]
MOMP/M^S/C-1	−5.35 ± 0.78[b]	1875 ± 418[b]	0.496 ± 0.017[b]	1.6 (BLD – 21.5)[k]
Ng-PorB/P/C-1	−17.41 ± 1.71	12,620 ± 2160	0.349 ± 0.011	442 (0.52 – 4366)
Ng-PorB/M^S/C-1	−10.62 ± 1.39	11,557 ± 2285	0.358 ± 0.01	44.9 (0.46 – 436.6)
MOMP/P/C-2	−6.03 ± 1.50[c]	584 ± 151[c]	0.809 ± 0.032[c]	0.66 (0.37 – 177.6)[l]
MOMP/M^S/C-2	−5.11 ± 1.01[d]	201 ± 40[d, g]	0.674 ± 0.039[d, g]	0.14 (0.02 – 146)[m]
Ng-PorB/P/C-2	−19.57 ± 1.93	5736 ± 1872	0.286 ± 0.013	125.8 (3.6 – 5161.5)
Ng-PorB/M^S/C-2	−15.43 ± 1.59	2686 ± 429	0.298 ± 0.01	94.4 (7 – 3792.5)
EB	0.19 ± 0.66	94.4 ± 11.9	2.315 ± 0.083	BLD (BLD – 0.00045)
MEM-0	−22.31 ± 1.62	1929 ± 315	0.241 ± 0.020	209 (24.8 – 2442)

^, BWC: body weight change;

*, BLD: below limit of detection.

[a], $p < 0.05$ by the Student's *t*-test compared with the group immunized with Ng-PorB/P/C-1.

[b], $p < 0.05$ by the Student's *t*-test compared with the group immunized with Ng-PorB/M^S/C-1.

[c], $p < 0.05$ by the Student's *t*-test compared with the group immunized with Ng-PorB/P/C-2.

[d], $p < 0.05$ by the Student's *t*-test compared with the group immunized with Ng-PorB/M^S/C-2.

[e], $p < 0.05$ by the Student's *t*-test compared with the group immunized with MOMP/M^S/C-1.

[f], $p < 0.05$ by the Student's *t*-test compared with the group immunized with MOMP/P/C-2.

[g], $p < 0.05$ by the Student's *t*-test compared with the groups immunized with MOMP/M^S/C-1 or with MOMP/P/C-2.

[h], $p < 0.05$ by the Mann Whitney's *U*-test compared with the groups immunized with Ng-PorB/P/C-1 or with MOMP/P/C-2.

[i], $p = 0.05$ by the Mann Whitney's *U*-test compared with the group immunized with MOMP/M^S/C-1.

[j], $p = 0.069$ by the Mann Whitney's *U*-test compared with the group immunized with MOMP/M^S/C-2.

[k], $p < 0.05$ by the Mann Whitney's *U*-test compared with the group immunized with Ng-PorB/M^S/C-1.

[l], $p < 0.05$ by the Mann Whitney's *U*-test compared with the group immunized with Ng-PorB/P/C-2.

[m], $p < 0.05$ by the Mann Whitney's *U*-test compared with the group immunized with Ng-PorB/M^S/C-2.

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Table 3

Pairwise Pearson's correlation assay between the observations.

Correlation	T-cell proliferation (cpm)	IgA in lungs (OD_{405nm})	IFN-γ in lungs (log of pg/ml)	D10 BWC* (%)	No. of Cm IFU in lungs
T-cell proliferation (cpm)	---	0.0006807	0.0029899	0.0007669	0.0000024
IgA in lungs (OD_{405nm})	---	---	0.0099292	0.0112737	0.0011700
IFN-γ in lungs (log of pg/ml)	---	---	---	0.0104718	0.0008410
D10 BWC* (%)	---	---	---	---	0.0011100
No. of Cm IFU in the lungs	---	---	---	---	---

*BWC: body weight change.

p value conducted by pairwise Pearson's correlation analysis between the in vitro proliferative response (cpm) of splenic T-cells before challenge, the levels of IgA and IFN-γ detected in the lungs, the percentage in body weight change, and the number of *C. muridarum* IFU recovered from the lungs at day 10 p.c. The statistical significant level is set to 0.05. All the *p* values showed the statistical significance in the pairwise Pearson's correlation analysis.